HAEMOSTATIC FACTORS IN DIABETES MELLITUS WITH PARTICULAR REFERENCE TO THROMBOTIC DISEASE

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V O L U M E I

C O N T E N T S

CONTENTS (continued)

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ABSTRACT

The aim of this Thesis was to investigate haemostatic function in diabetes mellitus and to relate abnormalities to the presence of micro- and macrovascular disease. Since poor metabolic control appears to favour the development of diabetic vascular complications, the influence of hyperglycaemia on haemostatic function was also studied.

In Part I, evidence that thrombosis is involved in the pathogenesis of micro- and macroangiopathy was discussed and the physiology of platelets and of the coagulation and fibrinolytic systems, with particular reference to their respective roles in thrombus formation, was reviewed.

In Part II, platelet aggregation and a battery of tests of coagulation and fibrinolysis were performed on 34 patients attending a diabetic out-patient clinic. Compared with 34 non-diabetic controls, significantly higher levels of fibrinogen, factors II, V, VII, VIII:C, α_2-macroglobulin and c_I-esterase inhibitor and lower levels of factor XI and antithrombin III were found, though the threshold concentrations of ADP, adrenaline and collagen required to produce second phase platelet aggregation were similar in diabetics and controls.

The refinement of a new, non-radioisotopic method for the measurement of platelet survival was described in Part III. Using this method, platelet survival of I2 diabetics was found to be significantly shorter than that of I2 controls. Diminished platelet survival may result from alterations in the platelet membrane; further evidence for membrane abnormalities in platlets from diabetic subjects was provided by the observation that platelets from 8 patients with severe retinopathy had significantly increased ^{125}I-fibrinogen binding compared with 9 non-diabetic controls, fibrinogen binding of platelets from 8 diabetics with slight retinopathy being intermediate between the two groups. Similarly, ADP-induced first phase platelet aggregation was found to be significantly increased in the group with severe retinopathy compared with the controls. Sensitivity of the platelets to ADP was found to be significantly correlated with the HbA_I concentration, but not with the degree of fibrinogen binding, suggesting that platelet aggregation and fibrinogen binding were related to the extent of microvascular disease,

but that aggregation may also be increased by poor metabolic control.

In Part IV, the effect of diabetic serum on the production of PGI_2 by thrombin stimulated cultured human vascular endothelial cells was assessed. Both PGI_2-like activity and 6-keto-$PGF_{I\alpha}$ concentrations were significantly lower in cells cultured in 20% serum from diabetics with proliferative retinopathy compared with non-diabetic controls, with a significant correlation between the depression in 6-keto-$PGF_{I\alpha}$ production and the concentration of HbA_I. Production of 6-keto-$PGF_{I\alpha}$ was not affected by changes in glucose concentration in the culture medium, and there were no significant differences when cells cultured in serum from well controlled diabetics were compared with serum from poorly controlled diabetics in the absence of vascular disease.

In Part V, effects of metabolic control on haemostatic function were studied. Compared with the results after stabilization of the diabetes, II patients with ketoacidosis had significantly raised levels of factors VIII:C and VIIIR:Ag and fibrin degradation products, a shorter partial thromboplastin time and reduced concentrations of antithrombin III. These changes may indicate vascular damage and intravascular fibrin deposition. Out of three deaths, two diabetics with the hyperosmolar syndrome had evidence of disseminated intravascular coagulation. In a second study, the effect of control of hyperglycaemia on haemostatic function was observed in I4 non-insulin dependent diabetics. After 2 months treatment with diet alone, II were given the sulphonylurea gliclazide, the others remaining on diet only. Compared with pretreatment values, significant reductions in platelet retention, factors VIII:C and VIIIR:Ag and plasma heparin neutralizing activity accompanied a fall in the plasma glucose concentration due to diet alone or diet plus gliclazide.

This Thesis has shown that diabetes mellitus is associated with a number of alterations of the haemostatic system. These changes appear to indicate tissue, and particularly vessel wall, injury and may in part be reversed by improved metabolic control. It remains uncertain whether such alterations predispose to diabetic vascular disease, though clinical trials of antithrombotic drugs during diabetic coma and in the long term prevention of micro- and macroangiopathy in diabetic patients may be indicated.

PART I INTRODUCTION

SECTION I DIABETES MELLITUS AND VASCULAR DISEASE

DIABETES MELLITUS AND VASCULAR DISEASE

Diabetes mellitus is not a modern disease. The earliest description may be contained in the Ebers papyrus (Egypt I500 BC), though the description of polyuria is not specific for diabetes mellitus. The first to detail the other acute symptoms and signs of the condition was the Arab physician, Aretreus of Cappodokia (2nd Century AD) and eight centuries later another Arab physician, Avicenna (967 - I037 AD), described the association of diabetes and gangrene. Diabetes was also recognized by the 3rd Century AD in both China and Japan and in the Indian Vedic literature of Sushrutha (5th Century AD). These historical aspects have been reviewed by Papaspyros (I964).

No mention of diabetes appeared in European literature until Thomas Willis (died I675) wrote of the "diabetes or pissing evil" (Malins, I968) and it was not until I852 that Marchal rediscovered diabetic gangrene.

Prior to the introduction of insulin treatment, diabetes was an incurable and largely untreatable disease with a high mortality, the majority dying rapidly from ketoacidosis or septicaemia (Marks, I97I). Records of patients attending the Joslin Clinic confirm this pessimistic outlook. Between I9I4 and I922 the mortality rate of 30 year-old diabetics was 236.8/I000 per year but the start of insulin therapy in I922 resulted in a dramatic fall in annual mortality to 74.8/I000 between I922 and I926 (Marks, I97I).

However, despite the undoubted improvements in the management of diabetic patients since the I920's, it has been apparent for many years that the life expectancy of diabetics is reduced compared to the non-diabetic population, and may not have significantly improved over the last thirty or forty years (Kessler, I97I). A number of careful studies have confirmed that the age and sex-specific death rates of diabetics compared to non-diabetics is significantly higher at virtually all age groups (Hayward & Lucena, I965; Hirohata, I967; Garcia et al., I974).

In the West, it is well-established that the commonest cause of this increased mortality is atherosclerosis and its complications (Kessler, I97I; Garcia et al., I974). In addition to macrovascular disease, microangiopathy, in the form of chronic renal failure,

is now the commonest cause of death in diabetics developing the disease before the age of 20 years (Knowles, I974; Kussman et al., I976).

In addition to this excess mortality, the management of diabetes mellitus and its vascular complications places a considerable burden on the social, financial and medical resources of the community. As an example, the United States National Health Interview Study in I965 estimated that over half a million diabetics were disabled in some way by their disease and that the total cost from loss of earnings, hospital admissions, out-patient attendances and drugs was almost two billion dollars/year (Knowles et al., I976).

The two main long-term complications of diabetes are atherosclerotic vascular disease and microvascular disease. Though there is evidence that thrombosis may be involved in the pathogenesis of both diseases, their pathology and epidemiology appear to be different.

ATHEROSCLEROSIS AND DIABETES MELLITUS

Information on the prevalence of large vessel disease in diabetes comes from three main sources : death certificates, autopsy series and epidemiological studies. All three have considerable limitations. Death certificates are frequently inaccurate and it has been shown that up to one third of known diabetics have no mention of the disease on their certificates (Cameron, I966). Autopsy series are highly selective and diabetes is difficult to diagnose after death if not suspected previously. In addition, pathological evidence of arterial disease is difficult to quantify and correlates poorly with its clinical manifestations. Epidemiological studies are not immune from criticism. Definitions of diabetes or abnormal glucose tolerance have varied widely. Many studies have been retrospective, with the problem that duration of diabetes is frequently uncertain since the onset of the disease may be insidious. In a review of I49 papers related to the vascular complications of diabetes, only eight had followed up a cohort assembled at the time that the diagnosis of diabetes was made. Most have been studies of the prevalence of complications for the available survivors of an undefined initial population, making results difficult to interpret (Kaplan & Fernstein, I973). However, recent carefully-conducted epidemiological studies such as the Framingham (Garcia et al., I974) and

Whitehall studies (Fuller et al., I980) have provided additional information confirming the excess mortality and morbidity from cardiovascular causes in diabetes.

The frequency and severity of cardiovascular disease in populations vary widely between different georaphical and ethnic groups (Keys, I970). Both autopsy and epidemiological surveys have shown that the prevalence of cardiovascular disease, though consistently higher in diabetics than non diabetics, follows the same pattern as in non diabetics (Robertson & Strong, I968; Keen & Jarrett, I979).

Reasons for the excess cardiovascular disease in diabetics remain obscure. Since diabetes is frequently accompanied by cardiovascular risk factors such as hypertension, hyperlipidaemia and obesity, doubt has been expressed as to whether diabetes is an independent risk factor for arterial disease (Royal College of Physicians, I976). However, recent evidence from the Framingham study favours the view that the association of known cardiovascular risk factors in diabetes does not satisfactorily account for the excess mortality and morbidity (Kannel & McGee, I979).

Ischaemic heart disease

Janeway (I9I6) and Levine (I922) were the first to record the association of diabetes and ischaemic heart disease. This impression was subsequently confirmed by many autopsy (Root et al., I939; Goldenberg et al., I958) and clinical studies (Bryfogle & Bradley, I957). Many of these studies may be criticized on methodological grounds, but modern prospective population surveys have essentially confirmed their findings (Garcia et al., I974; Fuller et al., I980). They also show that the normal male preponderance of ischaemic heart disease is not observed in diabetics where men and women have the same prevalence. The reason for the increased risk in diabetic women is unknown.

Diabetics have a higher mortality following myocardial infarction than non diabetics (Jarrett, I96I; Soler et al., I975). The connexion between diabetes and ischaemic heart disease is further suggested by the finding that patients with a proven myocardial infarction have a high prevalence of abnormal glucose tolerance tests (Sowten, I962; Bengtsson et al., I973) which persists for months after the acute event. There is now evidence that patients with only mild glucose intolerance have an increased risk of ischaemic heart disease. The Whitehall study has shown that men with impaired glucose tolerance

(defined as a 2h blood glucose following a 50g glucose load of 5.4 to 6.I mmol/l) have approximately double the risk of death from ischaemic heart disease as subjects with strictly normal glucose tolerance (Fuller et al., I980).

Peripheral vascular disease

The prevalence of clinical vascular disease in the lower extremities (generally defined as intermittent claudication, absence of pulses and gangrene) is considerably higher in diabetics than non diabetics (Kramer, I932; Bryfogle & Bradley, I957), and conversely, the frequency of diabetes among patients with peripheral vascular disease is higher than the prevalence of diabetes in the general population (Bell, I957; Gensler et al., I965). The reported high prevalence of gangrene in these studies could have been due to infection in the pre-antibiotic era, though more recent surveys confirm that peripheral vascular disease is still commoner in the diabetic (Kahn et al., I97I; Garcia et al., I974).

Cerebrovascular disease

Autopsy studies have suggested that atherosclerotic cerebrovascular disease is more common in diabetes (Baker et al., I96I; Grunnet, I963). The Framingham study has clearly shown an increased mortality and morbidity from cerebrovascular accidents in diabetics (Garcia et al., I974), though in a cohort study from the Joslin Clinic suggested that the increased risk of cerebrovascular accidents was confined to women only (Kessler, I97I). These studies make no distinction between thrombotic and haemorrhagic strokes. Since diabetics appear to have a higher prevalence of hypertension than non diabetics (Pell & D'Alonzo, I967; Garcia et al., I974), a proportion of the increased cerebrovascular risk is likely to be due to hypertensive rather than occlusive mechanisms.

ARTERIAL DISEASE

The Pathogenesis of Arterial Thrombosis

Thrombosis has been considered as the response of the vessel wall to injury (Mustard & Packham, I977), this response varying according to the type of vessel involved. In arteries, a "white thrombus" is formed, consisting mainly of aggregated platelets enmeshed in fibrin (Poole & French, I96I; French, I969). Venous thrombi have a much smaller platelet contribution but are largely made up of erythrocytes, leucocytes and fibrin (Chandler, I969; Paterson, I969).

The initiating event in arterial thrombosis appears to be endothelial damage, since a normal vascular endothelium is essentially non-thrombogenic (Nalbandian & Henry, I978). Experimentally, thrombosis can be induced by injuring the vessel wall by a variety of mechanical, electrical and chemical insults, but the mechanisms whereby the endothelium is damaged in vivo is uncertain. Mechanical stress, secondary to the repeated passive distension and contraction of arteries - "vessel fatigue" - has been invoked as a possibility (Duguid, I976). Studies of extracorporeal shunts in pigs (Murphy et al., I962; Mustard et al., I962) have shown that deposition of thrombi occur principally at sites of turbulence. Collisions between platelets in these areas may cause release of proteolytic enzymes and permeability factors which results in endothelial damage (Mustard et al., I965; Jorgensen et al., I972, I973). The development of fissures in an atheromatous plaque may also provide a thrombogenic surface (Friedman & Van den Bovenkamp, I966). Primates have been shown to develop endothelial lesions when fed high cholesterol diets (Vesselinovitch & Getz, I974) or made homocysteinaemic (Harker et al., I974). In addition, testosterone has been shown to cause damage to rabbit endothelium (Gaynor, I973), but the relevance of all these studies to human pathology is uncertain.

Arterial Thrombosis and Atheroma

Atheroma secondary to thrombosis

Rokitansky (I852) was the first to suggest that atheroma might result from the deposition of blood elements. This view was vigorously attacked by his contemporaries, notably Virchow (I860).

Rokitansky's "encrustation" theory was revived by Duguid (I946; I948) who provided evidence that atheromatous plaque formation was the consequence of mural thrombi which became incorporated in the vessel wall and organized to cause intimal thickening. Evidence from animal experiments and detailed human pathological studies have tended to confirm Duguid's thrombogenic theory (Roberts & Ferrans, I976; Woolf, I978).

On the basis of animal studies and tissue cultures of smooth muscle cells, Ross and Glomset (I976) have proposed a mechanism whereby endothelial damage leads to the formation of an atheromatous plaque. It is suggested that repeated endothelial damage results in platelet adhesion, with subsequent aggregation. Platelets release a mitogenic factor, characterized as a polypeptide of I3000daltons molecular weight (Antonaides et al., I975; Busch et al., I976). This factor appears to be very similar to a polypeptide isolated from bovine pituitary glands (Gospodarowicz, I974), and triggers the proliferation of cultured smooth muscle cells (Rutherford & Ross, I976). The importance of platelets in atherogenesis is suggested by the fact that experimental animal models do not form atheromatous lesions if the animals are made thrombocytopenic (Friedman et al., I976) or given antiplatelet agents such as dipyridamole (Burns et al., I976). Other smooth muscle mitogens such as low-density lipoprotein (Fischer-Dzoga & Kuo, I976), insulin (Stout et al., I975) and growth hormone (Ledet et al., I976) may also be important in stimulating smooth muscle cell accumulation in the arterial intima. It may be that the platelet-derived mitogen is the "trigger" for smooth muscle cell proliferation, with other factors playing a supportive role (Ross & Glomset, I976). In culture, smooth muscle cells have been shown to produce connective tissue proteins (Ross & Klebanoff, I97I) and glycosaminoglycans (Wright & Ross, I975), whch appear to be able to bind low-density lipoproteins (Iverius, I972). In human biopsy specimens, atheromatous plaques have been shown to contain fibrinogen and fibrin, presumably derived from organized thrombus, which is also capable of binding low-density lipoproteins (Smith & Staples, I98I).

It therefore seems possible that endothelial damage is followed by platelet adhesion and aggregation. Release of the platelet mitogen results in proliferation of smooth muscle cells in the arterial intima, followed by the production of connective tissue and glycosaminoglycans and accumulation of lipoproteins to give an atheromatous plaque (Ross & Glomset, I976).

Thrombosis secondary to atheroma

Thrombosis can cause arterial occlusion by several mechanisms. Accumulation of mural thrombus can eventually result in a complete blockage of the artery (Mitchell & Schwartz, I965). Alternatively, mural thrombi may fragment, releasing a shower of platelet microthrombi into the circulation. This possibility has been explored by Jorgensen et al.(I967), who found that infusion of adenosine-diphosphate into the coronary arteries of pigs resulted in the formation of platelet aggregates, and 36% of the animals died from ventricular fibrillation. Platelet microemboli have been directly observed in the retinal circulation of a few patients with transient ischaemic attacks (Ross Russel, I96I), but other aterial diseases are impossible to observe directly, and as Mitchell (I98I) has pointed out, many arterial conditions which present clinically as stroke, peripheral disease and ischaemic heart disease may be unrelated to thrombosis. The area of ischaemic heart disease has provoked the most controversy. Jorgensen et al. (I968) studied the pathology of patients dying suddenly secondary to presumed coronary artery disease and concluded that a large number of cases showed no signs of occlusive thrombi. Roberts and Buja (I972) found occlusive thrombi in over half of patients dying after myocardial infarction, but in only 8% of those who died suddenly. On the other hand, Haerem (I972; I974) was able to demonstrate microthrombi in the epicardial arteries of many patients dying suddenly of coronary artery disease.

A further possible mechanism of sudden coronary death is that transient platelet aggregation results in release of thromboxane A_2, causing vasoconstriction with resultant myocardial ischaemia precipitating a fatal arrythmia. Patients with unstable angina have been reported to have relatively high concentrations of thromboxane B_2 (the metabolite of thromboxane A_2) in coronary sinus blood (Hirsh et al., I98I), but this rise may follow, rather than be responsible for, the ischaemic episode (Robertson et al., I98I).

In summary, it would appear that the haemostatic system, and platelets in particular, play an important role in the initiation of atheroma, and may be responsible for the later occlusive episodes in an undefined proportion of cases. However, though it is accepted that platelets are important mediators of thrombosis, it is still uncertain whether in vitro hyperactivity results in an increased risk of thrombosis in vivo.

MICROANGIOPATHY

The existence in diabetics of degenerative changes involving retina, kidney and nerves was recognized during the second half of the I9th Century (McMillan, I975). However, it was not until the introduction of insulin treatment, resulting in the prolongation in life of young diabetics, that large numbers of diabetics were affected by these complications. The important concept of a disease of small blood vessels specific for diabetes was first formulated by Lundbaek (I953) and involvement of the microcirculation in the retinal, renal and neurological sequelae of diabetes was emphasized by the introduction of the term "microangiopathy" by Ditzel and Rooth (I955).

Microvascular disease now accounts for a large proportion of the mortality and morbidity of diabetic patients. Diabetic renal disease is now the commonest cause of death in almost half the patients developing diabetes before the age of 20 (Knowles, I974) and in the West, diabetes is the commonest cause of blindness in adults under the age of 65 (Kahn & Miller, I974). Microvascular disease also contributes significantly to diabetic peripheral vascular disease (Christensen, I972) and may also affect the capillaries supplying the myocardium resulting in a form of cardiomyopathy (Ledet et al., I979).

The pathological basis of microangiopathy was established by light microscopic studies which demonstrated the characteristic renal lesion of diabetes - glomerular nodular sclerosis - by Kimmelstein and Wilson (I936) and the presence of PAS-positive material in the walls of capillaries in retinal (Friedenwald, I949) and renal (McManus, I948) specimens from diabetic patients. Shortly after the introduction of the electron microscope, the vessel wall thickening was attributed to an increase in the capillary basement membrane (Farquar et al., I959). Basement membrane thickening has tended to dominate the thinking behind diabetic microangiopathy ever since.

In view of the very different pathological appearances in end-stage renal and retinal disease secondary to diabetes, and the possible different aetiologies and precipitating factors influencing diabetic small vessel disease in the various vascular beds of the body, the use of the term "microangiopathy" to describe possibly quite different processes has been questioned (Winegrad & Greene, I978). However, basement membrane thickening has been described in almost all small vessels in diabetic

subjects (Reddi, I978), and capillary microaneurysms are not restricted to the retina, but have also been observed in the glomeruli (Bloodworth, I978) and the myocardium (Factor et al., I980) of diabetics. Further support for a unitarian view of diabetic small vessel disease comes from a number of epidemiological studies which show that the prevalence of clinically apparent retinopathy, nephropathy and neuropathy are strongly correlated and all three are closely related to the duration of diabetes (Pirart, I978; Jarrett & Keen, I979; West et al., I980).

Aetiology and pathogenesis of diabetic microangiopathy

In spite of intensive histological and histochemical studies over the past three decades, the cause of the characteristic lesions of diabetes and the relationship between these pathological findings and the clinical manifestations of diabetes remains unclear. The prolonged controversy and sometimes acrimonious debate concerning the stage in the evolution of diabetes that basement membrane thickening occurs (Gundersen et al., I978; Siperstein et al., I978) has tended to obscure the fact that so little is known of the early functional changes in the diabetic microvasculature, and of the sequence of events leading to the loss of an eye or renal failure.

Any theory of the aetiology of diabetic microangiopathy must reconcile a number of apparent paradoxes. There is now compelling epidemiological evidence that when asymptomatic individuals with abnormal glucose tolerance are observed over a number of years, retinopathy is very rarely observed if the 2hr blood glucose level is less than II.I mmol/l (Sayegh & Jarrett, I979; Rushforth et al., I979). This is in marked contrast to ischaemic heart disease, where even impaired glucose tolerance (5.4 - 6.I mmol/l) results in a two-fold increase in mortality (Fuller et al., I980). In contradistinction to ischaemic heart disease, there are only small national differences in nephropathy and retinopathy prevalence suggesting that environmental factors may be less important in microangiopathy (Jarrett & Keen, I979). The relationship between metabolic control and the vascular complications of diabetes is a subject for a separate thesis, but currently a consensus of opinion favours the view that good control of blood glucose levels reduces the prevalence of microvascular disease. Evidence in support of this view has recently been extensively reviewed by Pirart (I978), Tchobroutsky (I978) and Skyler (I979).

On the other hand, microvascular complications are not inevitable consequences of diabetes, even after years of mediocre metabolic control. It is well documented that 20 - 40% of patients surviving more than 40 years of diabetes have little or no clinically important evidence of microangiopathy (Oakley et al., I974; Paz-Guevara et al., I975). After I5 years of diabetes, 60 - 70% of patients have retinopathy but only 5% develop maculopathy (background retinopathy with oedema of the macula) and 3 - I0% proliferative retinopathy, the two main causes of blindness in diabetes (Blakenship & Skyler, I978) and 59% of a series of 40 year survivors had no noteworthy renal disease (Paz-Guevara, I975), raising the possibility that only a subset of patients are at risk of developing severe complications. Unfortunately, at present there is no means of identifying this high risk group.

Apart from the duration of diabetes, hypertension appears to favour the development of retinopathy (Jarrett & Keen, I979; West et al., I980) though whether it accelerates the progression from background to proliferative retinopathy is not known (West et al., I980). Though there is a tendency for twins with diabetes to have the same extent of retinopathy (Pyke & Tattersall, I973) and certain HLA loci have been reported to occur more commonly in diabetics with proliferative retinopathy (Dornan et al., I98I) the role of heredity in microvascular complications remains undetermined.

Growth hormone has been implicated in the pathogenesis of microvascular disease, notably by the group of Lundbaek(I976). The beneficial effect of pituitary ablation on severe proliferative retinopathy, and the finding of abnormal growth hormone secretion in insulin-dependent diabetics suggest that this hormone could have a direct effect on the micocirculation in the presence of hyperglycaemia.

Though organs such as eye and kidney have totally different functions, the early changes of diabetes appear to be remarkably similar. Direct observation of retinal and conjunctival vessels shows initially dilatation of venules (Ditzel & Moinat, I959). The stimulus for this dilatation is not known but hypoxia has been proposed as one possibility. At this early stage, retinal blood flow is increased, this abnormality being partially reversible by tight metabolic control (Kohner, I976). In addition, there is a breakdown of the blood-retinal barrier as demonstrated by leakage of fluorescein into the vitreous (Cunha-Vaz et al.,

I978). Later changes include irreversible arteriolar constriction (Ditzel & Moinat, I959), followed by sclerosis of capillaries, arterioles and venules (Ashton, I949). At this stage, areas of non-perfusion of the retina may be observed on fluorescein angiography (Kohner, I976), and microaneurysms and hard exudates with dot and flame haemorrhages develop to give the appearances described as background retinopathy (Blakenship & Skyler, I978). The cause of the areas of capillary non perfusion is unknown, though microthrombus formation is one factor which may favour retinal ischaemia. Retinal hypoxia is believed to be a strong stimulus for new vessel formation(Blakenship & Skyler, I978) so that capillary occlusion appears to be of critical importance in the pathogenesis of proliferative retinopathy.

The morphology of the kidney is less easy to observe than the retina. The earliest functional change appears to be an increase in glomerular filtration rate, reversible by insulin therapy (Morgensen et al., I979). The glomerular filtration rate remains above normal until the onset of proteinuria when it descends to the normal range before falling below normal with the onset of renal failure (Morgensen et al., I979). As with the increased retinal blood flow, the cause of the increased glomerular filtration rate is unknown, though an increase in the surface area of the glomerulus may be involved (Morgensen et al., I979). Subsequently, the afferent glomerular arterioles undergo arteriosclerosis (Bell, I953) followed by diffuse and then nodular glomerulosclerosis (Farquar et al., I959).

Similar changes of initial venous dilatation, followed by arteriolar constriction and sclerosis of small blood vessels has also been described in the skin of diabetic patients (McMillan, I975).

Though neuropathy is linked in time with the appearance of retinopathy and nephropathy it is less certain that a vascular aetiology underlies the the various neuropathic syndromes of diabetes, where metabolic factors may be as important (Thomas& Ward, I975). Nonetheless, the finding of fibrin-plugged vessels supplying sural nerves taken from patients with evidence of diabetic neuropathy suggests that neuronal ischaemia may be involved in certain neuropathies (Timperley et al., I976).

GLYCOSYLATION OF HAEMOGLOBIN

Until recently, assessment of long term glycaemic control of diabetic patients has been severely limited. Determination of urinary glucose concentrations is notoriously unreliable and problems of sampling blood glucose concentrations sufficiently frequently to obtain an accurate estimation of overall blood glucose control over a prolonged period of time seemed intractable. This problem has been greatly simplified by the observation that the concentration of glycosylated haemoglobins correlates with mean blood glucose levels. Haemoglobin A_{Ic} (HbA_{Ic}) is the most abundant minor component of human haemoglobin A, normally comprising approximately 4% of the total haemoglobin. Interest in HbA_{Ic} was stimulated by the discovery by Rahbai (I968) that this component was two to three times higher in diabetic compared with non diabetic subjects. The biosynthesis and chemical structure of HbA_{Ic} have recently been reviewed by Bunn (I98I). Glucose first condenses with the N-terminal amino group of the β-chain of haemoglobin to form a Schiff base (pre-HbA_{Ic}). Pre-HbA_{Ic} is unstable and can either dissociate to HbA and glucose or undergo an Amadori reaction to form a more stable ketoamine linkage (HbA_{Ic}). This almost irreversible reaction proceeds slowly and continuously throughout the lifespan of the erythrocyte, the rate being dependent on the blood glucose concentration. Other minor components: HbA_{Ia}, HbA_{Ib}, HbA_{Ia_1} and HbA_{Ia_2} are also glycosylated but are quantitatively less important than HbA_{Ic}.

A number of methods have been developed to measure glycosylated haemoglobin. HbA_{Ic} elutes more rapidly than HbA on cation exchange chromatography. Several minor components of haemoglobin (HbA_{Ia},HbA_{Ib}) elute faster than HbA_{Ic}. When the total fast fraction (HbA_{Ia}, HbA_{Ib}, HbA_{Ic}) is measured together, it is referred to as HbA_{Ia-c} or more simply, HbA_{I}. A number of column chromatographic methods of varying degrees of complexity have been devized (reviewed by Bunn, I98I), but for routine clinical use, provided that the pH of the elution buffers and the temperature are carefully controlled, the microcolumn method (which measures HbA_{I}) gives satisfactory results (Abraham et al., I978). This is the method employed in Parts III and IV of this Thesis.

Other methods including isoelectric focussing (Beccaria et al., I978) and a colorimetric method (Fluckiger & Winterhalter, I976) have also been developed, but column methods remain the most popular.

A number of reports have confirmed the utility of HbA_{Ic} and HbA_{I} measurements in the assessment of glycaemic control in diabetic patients (Koenig et al., I976; Ditzel & Kjaergaard, I978; Boden et al., I980). These reports indicate that HbA_{I} concentrations correlate well with glycosuria and mean blood glucose measurements. When poor metabolic control is improved, HbA_{I} levels fall towards normal, with a lag period of 4 - 6 weeks before a plateau is reached, though there is evidence that levels can rise more rapidly if a period of good control is followed by high blood glucose levels (Boden et al., I980).

Measurement of glycosylated haemoglobin has therefore provided a useful tool, not only for the management of diabetic patients, but also for the investigation of the relationship between glycaemic control, other haematological abnormalities and the development of vascular complications in diabetes.

It is not known whether increased glycosylation of haemoglobin alters erythrocyte function in diabetes. Though the addition of 2,3-diphosphoglycerate causes much less of a reduction in oxygen affinity of HbA_{Ic} compared to that of HbA (Bunn & Briehl, I970), the oxygenation of intact erythrocytes appears to be only slightly affected by alterations in HbA_{Ic} (Bunn, I98I).

Non-enzymic glycosylation is not exclusive to haemoglobin, but is likely to affect many other proteins. Recently, glycosylation of albumin (Guthrow et al., I979), fibrinogen (McVerry et al., I980a) erythrocyte membrane protein (Miller et al., I980), collagen (Rosenberg et al., I979) and crystalline lens protein (Pande et al., I979) have been described in diabetic patients. Whether such biochemical modifications result in altered function is currently under study. One such experiment has shown that when glycosylated plasma proteins were injected into mice, thckening of the glomerular basement membrane resulted compared with animals injected with non-glycosylated proteins (McVerry et al., I980b).

SECTION 2 PLATELETS

PLATELETS

HISTORICAL

The existence of red cells was recognized by the I7th centuary, but it is doubtful whether platelets were identified until chromatic aberration of the early compound microscopes was overcome by Johseph Lister in I826 (Robb-Smith, I967). Gulliver (I842), in an appendix to his translation of the works of Gerber, described spheres of 2.5 micron in diameter which were almost certainly platelets. He observed that these particles adhered to white cells and developed granules on standing, but believed that they were not involved in fibrin formation. In the same year, Addison (I842), examining coagulating blood, noted that "the fluid, i.e. liquor sanguis, contained a great number of minute molecules or granules". He suggested that they arose from broken-down white cells and that they could be important in the generation of fibrin. Also in I842, Donné described three blood particles: red cells, white cells and "des globulins du chyle", the latter being described as I/300 mm in diameter. Since he used water as a diluent, it is uncertain that the droplets of chyle were indeed platelets (Robb-Smith, I967).

The existence of a third particle smaller than red and white cells was confirmed by Simon (I844) who described them as molecules of fibrin and Zimmerman (I846) who believed that they were red cell precursors. Wharton Jones (I850) made a detailed study of the blood vessels of the frog's foot in response to inflammation. He found that following injury to the vessel wall "grayish granulous-looking plugs, composed, apparently, of colourles corpuscles and coagulated fibrin, are sometimes to be seen to block up an artery". He was therefore probably the first to describe in vivo thrombus formation but, as frog platelets are nucleated, failed to identify the corpuscles as platelets, believing them to be white cells.

Beale (I864) considered fibrin to be derived from platelets "the production of the material we know as fibrin is due to the gradual death of minute particles of the living matter of the white, and small colourless corpuscles, which takes place under ordinary circumstances when blood escapes from the vessels of the living body". Osler (I874) described platelet clumping and pseudopod formation but confessed "we know nothing of the origin and destiny of these corpuscles".

Hayem (I878) established the platelet as a separate entity

(l'hématoblaste), counted them reasonably accurately, recognized their role in clot formation and retraction but believed that they were red cell precursors. Progress during the next forty years was consolidated and amplified by the work of Bizzozero (I882). He damaged guinea pig blood vessels and showed that the initial stage in thrombus formation was platelet adhesion and aggregation to the site of injury. He was the first to use the term platelet (blutplätchen). These observations were extended by Eberth and Schimmelbusch (I886), who showed that platelet accumulation occurred before fibrin formation, and introduced the term "viscous metamorphosis" to describe the shape change induced by platelets in contact with a damaged blood vessel or foreign surface.

The platelet then fell into obscurity until Wright (I905) showed clearly that platelets were derived from a bone marrow precursor, the megakaryocyte. Pratt(I905) devised a more accurate method of counting platelets and Duke (I9I0) demonstrated the first haemorrhagic disorder secondary to a low platelet count.

A further fifty years was to elapse before the enormous growth in the understanding of platelet histology, biochemistry and physiology. In spite of these advances, the role of the platelet in thromboembolic disease is still not fully understood.

PLATELET STRUCTURE AND PHYSIOLOGY

The resting platelet is disc shaped, measuring 2 - 4 microns in diameter. Platelet ultrastructure has been extensively investigated by electron microscopy, notably by the group of White (White, I97I; White, I972; White & Gerrard, I976). The platelet membrane is a triple layered structure with an equatorial band of microtubules running round the perimeter close to the surface membrane, which appears to maintain the platelet's discoid shape. Intracellular structures include a surface connecting vesicular system (the open cannicular system) and a complex of dense vacuoles (the dense tubular system) which may represent remnants of the megakaryocyte endoplasmic reticulum (White, I972). Microfilaments are distributed throughout the cytoplasm, but seem to be connected to the wall of the surface of the external membrane, and are believed to be components of the actinomyosin-like contractile system ("thrombosthenin").

At least three types of membrane-bound granules can be demonstrated by electron microscopy. Alpha granules, containing fibrinogen,

β -thromboglobulin, platelet factor 4 and a mitogenic factor; dense bodies containing calcium, adenosine diphosphate, adenosine triphosphate and serotonin and lysosomal granules (shown with histological stains) containing acid hydrolases (White, I97I; Day & Holmsen, I97I; Bentfield & Bainton, I975). Mitochondria and glycogen granules are also present, equipping the cell for glycolysis and aerobic respiration.

Platelets are derived from megakaryocytes, which probably share a common origin with myeloid and erythroid cells (Becher et al., I963). Once mature, the megakaryocyte transforms from a spherical to a highly irregular shape (Behke, I969) and fragmentation of the cytoplasm produces 2,000 to 3,000 platelets. This phenomenon occurs when the ploidy number of the megekaryocytes is between 8 and 32 (Penington & Streatfield, I975), and it is thought that ploidy number at release is a feature governing size of the platelets(Penington & Streatfield, I975; Paulus I975). Platelets show a marked variation in volume and density in normal subjects (McDonald et al., I964; Karpatkin, I969) though it is still unclear to what extent dimunition of platelet size occurs during the life of the platelet is still unclear. The normal platelet count in man ranges from 250,000 - 500,000/ul and turnover is approximately 3.5 x 10^7/ml/day (Harker & Finch, I969). Platelets survive in the circulation under normal conditions for 9 - II days. Recent evidence suggests that platelets lose portions of their membrane glycoproteins whilst in the circulation (George et al., I976, I978) and loss of platelet surface sialic acid has been shown to accelerate platelet removal from the circulation (Greenberg et al., I975).

PLATELET FUNCTION

A diverse range of functions and properties have been attributed to platelets including storage of substances such as adenosine diphosphate and serotonin, adhesion, aggregation, procoagulant activity, contraction, secretion, prostaglandin synthesis and phagocytosis (Gordon & Milner, I976). The following brief review will concentrate on these properties which seem most relevant to the mechanisms of thrombosis.

Platelet Adhesion

Following the observations of Bizzozero (I882), Bounameau (I959) and Hugues (I960) realized that platelets adhered in vivo primarily to subendothelial connective tissue. Subsequent investigators established that platelets adhered to several components of the subendothelium

including collagen fibrils (Kjaerheim & Hovig, I962; Hugues & Lapière, I964), elastin (Hugues, I960) and blood vessel basement membrane (Baumgartner et al., I967).

In vivo adhesion to subendothelium is usually followed by platelet-platelet interactions (aggregation) leading to the formation of mural platelet thrombi or platelet plugs. Electron microscope studies of this process have shown that the participating platelets become degranulated (Kjaerheim & Hovig, I962). This degranulation was later found to correspond to the platelet release reaction (Grette, I962; Holmsen et al., I969) which is observed when platelets are aggregated in vitro.

Von Willebrand factor and platelet adhesion

The role of a plasmatic factor in platelet adhesion was first recognized following the discovery that the prolonged bleeding time found in von Willebrand's disease could be corrected by the transfusion of factor VIII concentrate (Nilsson & Holmberg, I979). The terminology and the molecular heterogeneity of the factor VIII:von Willebrand molecule are complex, and only a brief resumé can be given in this section. Von Willebrand factor has been the subject of a number of reviews (Bloom, I977; Koutts et al., I979; Bloom, I979). The human factor VIII:von Willebrand factor has two main biological activities: procoagulant activity (FVIII:C), the ability to correct the long clotting time of haemophilic plasma and von Willebrand activity (VIII:vWF), the ability to cause platelet retention in a glass bead column or to aggregate platelets in the presence of the antibiotic ristocetin. Material which precipitates with heterologous antisera, factor VIII-related antigen (VIIIR:Ag), is associated with the platelet-related activities (Koutts et al., I979; Bloom, I979).

The factor VIII molecule has proved difficult to purify and characterize. It is a glycoprotein with repeating covalently linked subunits of molecular weight 250,000 daltons. The size of the oligomer in vivo is not certain, but may be between 0.5 x 10^6 to 20 x 10^6 daltons, the larger oligomers seeming to be most active in promoting platelet adhesion (Bloom, I977).

Endothelial cells have been shown to produce VIIIR:Ag, but not VIII:C activity (Jaffe et al., I973; Tuddenham et al., I98I). This antigen is also found in platelets, and is released when they undergo

aggregation (Koutts et al., I978). In contrast, FVIII:C activity has never been convincingly been demonstrated in endothelial cells or platelets, but may be produced in the reticuloendothelial system (Bloom, I979).

When blood from patients with von Willebrand's disease is passed over de-endothelialized rabbit aortas, platelets adhere poorly, particularly at high shear rates, suggesting a role for VIII:vWf in promoting platelet adhesion (Weiss et al., I978). Specific receptors for VIII:vWf exist on the platelet membrane, and are activated in vitro by ristocetin (Kao et al., I979). Factor VIIIR:Ag, in addition to being present in the plasma, has recently also been demonstrated in the subendothelium of human umbilical vessels (Rand et al., I980) and Sakariassen et al. (I979) have shown that the adherence of platelets to de-endothelialized human arteries corresponded to the quantity of VIIIR:Ag bound to the subendothelium. However, the relative contributions of plasmatic, platelet, and subendothelial VIII:vWf to normal haemostasis is not known. Because of a lack of suitable models which satisfactorily measure increased platelet adhesion, it is not known whether high levels of factor VIII:vWf cause an increased risk of thrombus formation (Paton et al., I98I).

Platelet Aggregation

In I960, Hellem discovered that a substance (R factor) caused platelets to aggregate in vitro, this factor being identified as adenosine diphosphate by Gaarder et al.(I96I). Knowledge of platelet behaviour advanced rapidly with the invention of the platelet aggregometer (Born & Cross, I962), which enabled the turbimetric measurement of platelet aggregation under controlled conditions.

Platelets have been found to react to a diverse range of stimuli in a remarkably uniform manner, which has been termed the Basic Platelet Reaction or BPR (Holmsen, I976). The BPR may be divided into three phases: induction, transmission and execution.

Induction

Over fifty substances are capable of inducing platelet aggregation in vitro (Mustard & Packham, I970), though it is likely that relatively few are important in vivo. Platelets interact with a large number of these agents through specific receptor mechanisms (Mills & Macfarlane, I976). This diversity of receptor sites is provided by

the glycoprotein coat of the platelet membrane. Identification of functional glycoproteins has been facilitated by the study of patients with inherited bleeding disorders caused by a defect in platelet function. Thus, glycoprotein Ib (absent in Bernard-Soulier syndrome)is known to be required for the binding of FVIII:vWf and adhesion to subendothelium, and glycoprotein IIb and III (absent in Glanzmann's thrombasthenia) is required for platelet aggregation and clot retraction. The characterization of these functional glycoproteins has recently been reviewed by Nurden and Caen (I979) and Phillips (I980).

Agonists of platelet induction may be divided into two main groups: macromolecules such as thrombin, collagen and antibody-antigen complexes; and small molecular weight compounds such as ADP, adrenaline and serotonin (Mills & Macfarlane, I976).

Transmission

By a process that is still not fully understood, induction causes an alteration in the platelet membrane which results in the mobilization of a transmitter, which is almost certainly calcium, into the platelet cytosol (Holmsen, I976). The precise role of calcium is difficult to establish in spite of extensive studies of calcium flux into the cell, and of changes in intracellular calcium levels . This is because calcium is one of the constituents released by the dense bodies, and the quantity released is greatly in excess of the small changes in cytoplasmic calcium which probably regulate cell function (Crawford & Taylor, I977). Recent work suggests that one component of the platelet contractile system, myosin light chain,is phosphorylated by a specific protein kinase (Daniel & Adelstein, I976) which is activated by calcium through a calmodulin subunit (Hathaway & Adelstein, I979). Phosphorylation of the myosin stimulates actomyosin ATPase (Adelson & Conti, I973), resulting in contraction (Lebowitz & Cooke, I978). However, though myosin phosphorylation is strongly associated with the secretory process, it seems not to be associated with shape change and aggregation (Haslam & Lynham,I978).

Another important modulator of platelet reactivity is cyclic AMP. Agents which induce platelet aggregation tend to reduce cyclic AMP levels, while inhibition of aggregation increases levels of this agent (Haslam & Taylor, I97I; Salzman, I972). However, this is certainly an oversimplified view, since the ability of various agonists of platelet aggregation to reduce cyclic AMP levels does not parallel their efficiency

as aggregating agents (Robison et al., 1971; McDonald & Stuart, 1973). How cyclic AMP influences platelet aggregation is unknown, though regulation of cyclic AMP-dependant protein kinases may be important (Booyse et al., 1976; Steiner, 1976).

Execution

Influx of calcium into the cytosol results in the transformation of the platelet from a disc to a sphere with long cytoplasmic protrusions, the so-called platelet shape change (Born, 1970).
Shape change is an energy-requiring process (Holmsen et al., 1974), and permits interactions between similarly-stimulated platelets and the liberation of platelet granule contents, the process named the release reaction by Grette (1962). Day and Holmsen (1971) showed that release of dense body constituents induced by ADP, adrenaline or low concentrations of thrombin or collagen was not accompanied by release of **α** granules whereas higher concentrations of thrombin and collagen caused release of both granule constituents.

Products released by the platelet release reaction have important functions in haemostasis and thrombus formation. In addition to ADP, platelets also release thromboxane A_2, a powerful vasoconstrictor and platelet aggregating agent (see below). Permeability factors (Mustard et al., 1965) may result in endothelial cell injury (Jorgensen et al., 1972) and a mitogenic factor (Ross et al., 1974) are also liberated, and may be important in the initial stages of atherogenesis (Ross & Glomset, 1976). Fibrinogen and platelet factor 4 are also released, and platelet factor 3 is made available on the platelet surface, thus favouring coagulation (see below).

Platelets and Coagulation

Blood coagulates more efficiently when platelets are present, but this coagulant activity is present only after platelets have undergone the release reaction (Marcus, 1969). This activity has been termed platelet factor 3 (PF3) and appears to be related to phospholipids which are made available on the platelet surface during the release reaction (Marcus, 1969). PF3 activity is required for the activation of factor X by IXa, VIII and calcium and also in the reaction in which Xa, V and calcium convert prothrombin to thrombin (Walsh, 1974). There is also evidence that platelets are capable of activating factor XII (in the

presence of ADP) and of factor XI (in the presence of collagen), (Walsh, I974).

The platelet release reaction also results in the liberation of two platelet-specific proteins, β-thromboglobulin and platelet factor 4 (Moore et al., I975; Niewiarowski & Thomas, I969). Platelet factor 4 (PF4) has antiheparin properties which may be of importance during heparin therapy, but it could also interact with heparans produced by the vessel wall (Rosenberg, I975). The function of β-thromboglobulin is uncertain. It is related, though immunologically distinct from PF4 and recently Rucinski et al. (I979) have described another protein closely related to β-thromboglobulin, which they have named "low-affinity PF4." Sensitive radioimmunoassays have been developed for both PF4 and β-thromboglobulin, and these tests have been widely used as markers of the platelet release reaction in vivo (Zahavi and Kakkar, I980 ; Kaplan & Owen, I98I).

Prostaglandins, Thromboxanes, Platelets and the Vessel Wall

The realization that prostaglandins might play an important role in platelet reactions began with the observation that platelets produce PGE_2 and PGF_2 during aggregation (Smith & Willis, I970), and that this production was blocked by aspirin (Smith & Willis, I97I; Vane, I97I). Since aspirin had previously been shown to inhibit platelet aggregation (O'Brien, I968; Zucker & Peterson, I968), inhibition of prostaglandins seemed to provide an explanation of this phenomenon. However, PGE_2 and PGF_I were shown to have only minor effects on platelet aggregation(Shio & Ramwell, I972). Incubation of platelet microsomes was found to generate an unstable compound with potent pro-aggregatory properties which was named labile aggregation stimulating substance or LASS (Willis et al., I974).

The group of Samuelsson had previously demonstrated the existence of an enzme present in sheep vesicular gland preparations (cyclo-oxygenase) capable of converting arachidonic acid to cyclic endoperoxides and subsequently that platelets produced two highly unstable endoperoxides termed PGG_2 and PGH_2 which were potent inducers of platelet aggregation (Hamberg et al., I974a, I974b), the formation of which was blocked by aspirin (Hamberg & Samuelsson, I974). The cyclic endoperoxides were later found to rapidly be transformed into another labile substance,

thromboxane A_2, another powerful aggregating agent (Hamberg et al., I975), which appears to be more important than PGG_2 and PGH_2 in vivo (Smith et al., I976). The exact mode of action of thomboxane A_2 on platelet aggregation is uncertain, opinions differing as to whether it acts by potentiating the release of intraplatelet ADP (Zucker, I980) or whether it has a separate effect on intracellular calcium transport (Gerrard & White, I978). Thromboxane A_2 is also a powerful vasoconstrictor and is rapidly degraded into the less active compound, thromboxane B_2 (Hamberg et al., I975).

In I976, an arachidonic acid derivative produced by vessel wall preparations with potent platelet inhibitory and vasodilatory properties was identified (Moncada et al., I976, I977). Initially named PGX, the substance was finally called prostacyclin or PGI_2. Like thromboxane A_2, PGI_2 is formed from arachidonic acid by the action of cyclo-oxygenase, and this process is inhibited by aspirin (Moncada et al., I977).

The principal effect of PGI_2 appears to be an increase in cyclic AMP (Gorman et al., I977). Like thromboxane A_2 , PGI_2 has a short half-life in plasma, being degraded to the stable form, 6-keto-$PGF_{I\alpha}$, which has only minor effects on the inhibition of platelet function (Johnson et al., I976).

Following the discovery of PGI_2, the concept has arisen that this prostaglandin is the physiological medium that protects the vessel wall from the accumulation of platelet aggregates, and explains the relative non-thrombotic surface provided by the endothelial cell monolayer. It is suggested that, under normal circumstances, a delicate balance exists between platelet thromboxane production and vessel wall PGI_2 production, with an increase in thromboxane relative to PGI_2 production resulting in thrombus formation (Moncada & Vane, I979). Complete acceptance of this attractive hypothesis is hindered by two lines of evidence. Firstly, thrombin and collagen, likely to be important inducers of platelet aggregation in vivo, have been shown to be capable of aggregating platelets after inhibition of platelet cyclo-oxygenase, suggesting prostaglandin independent pathways of platelet aggregation (Zucker & Peterson, I970; Packham et al., I977). Secondly, congenital cyclo-oxygenase deficiency leads to only a mild bleeding disorder (Malmsten et al., I975) and ingestion of high doses of aspirin which block both platelet thromboxane and vessel wall prostacyclin

production result in a prolonged bleeding time (Preston et al., 1981), suggesting that in the absence of prostaglandins, the balance of haemostasis favours haemorrhage rather than thrombosis.

TESTS OF PLATELET FUNCTION

In comparison with the sophisticated techniques which have been used to study platelet biochemistry, physiology and pharmocology, tests currently used to assess platelet behaviour in clinical studies are relatively primitive. The majority of these tests are performed in vitro and generally measure platelet-surface interactions ("adhesion") or platelet-platelet interactions ("aggregation"), though measurements of platelet survival and plasma levels of certain platelet-specific proteins (β-thromboglobulin and PF4) have also been used to assess platelet function in thrombotic conditions.

The relation between these different tests and their relevance to thrombogenesis has not yet been clearly established; lack of standardization of these tests has made comparison of results between different laboratories difficult, and may explain many of the discrepancies which have been reported.

In Vitro Tests

Platelet adhesion

These tests have been divided into two groups : those which measure platelet adhesion alone, and those which measure adhesion-aggregation (Bowie& Owren, 1978). Tests of adhesion alone include exposure of blood or platelet-rich plasma to glass slides or cover slips, rotating glass tubes coated with collagen or other proteins whereas adhesion-aggregation is measured by exposing blood or platelet-rich plasma to rotating glass bulbs or by filtration through glass wool or glass bead columns. These methods are reviewed fully by Meyer (1972) and Bowie and Owren (1978). Largely because of its simplicity, the glass bead method has been most extensively. Native or anticoagulated blood, or platelet-rich plasma is passed through a plastic tube filled with glass beads of a standard size and the percentage of platelets retained in the column is calculated from the platelet counts before and after the filtration. The test is influenced by many factors such as haematocrit, anticoagulant, temperature, flow rate through the

and even the type of plastic used to make the column (Meyer, I972; Bowie & Owren, I978). In spite of these technical problems, there is general agreement that platelet adhesion (more correctly termed platelet retention) is reduced in patients with von Willebrand's disease. However, results in patients with thrombotic diseases are contradictory, both raised and normal retention having been reported (Meyer, I972). A notable exception is diabetes mellitus, where increased platelet retention has frequently been described. The significance of this finding will be discussed more fully in Part V , Section 2 .

Platelet aggregation

The most widely used test of platelet aggregation was first described by Born and Cross (I962) and O'Brien (I962). Citrated platelet-rich plasma is placed in a transparent cuvette and light transmission through the platelet suspension is recorded. Upon addition of an aggregating agent to the stirred platelet suspension, aggregate formation increases the light transmission in a manner proportional to the rate and extent of platelet aggregation. Different inducers of platelet aggregation produce different shapes of aggregation traces. For example, adenosine diphosphate and adrenaline produce single, reversible curves at low concentrations, but at higher concentrations of inducer, a biphasic response is obtained, the second irreversible phase being parallelled by the release reaction (Zucker & Peterson, I970). This second phase of platelet aggregation is only observed when the extracellular calcium concentration is low (e.g. citrated platelet rich plasma), and its in vivo relevance has been questioned (Mustard et al., I975; Macfarlane & Mills, I975). Arachidonic acid (Kinlough-Rathbone et al., I976) and ristocetin (Howard, I975) also are capable of aggregating platelets and inducing a biphasic response in the aggregometer. This second phase can be abolished by the addition of non-steroidal anti-inflammatory drugs such as aspirin and indomethacin by inhibition of platelet cyclo-oxygenase (Roth & Majerus, I975). When collagen is added to platelet-rich plasma, a lag phase is followed by a single aggregation curve, which is associated with release of adenosine diphosphate and thromboxane A_2 (Smith et al., I976).

Quantification of platelet aggregation curves has not been standardized. The majority of authors measure the maximum change,

the time taken to reach maximum change and the initial rate of change of light transmittence. However, a number of investigators have tried to analyze aggregation mathematically (Skoza et al., I967; Frojmovic, I978). Mathematical treatment of platelet aggregation curves will be discussed more fully in Part III, Section 2 of this Thesis.

Platelet aggregation, as assessed in the aggregometer, is influenced by a large number of technical factors such as size and shape of stir bars, temperature, concentration of calcium, pH and "temporal drift" - the variation in aggregability with time after blood sampling. In addition, smoking, excercise, mental stress and menstruation in women may cause aggregation curves to change markedly. These variables are reviewed extensively by Sixma, (I972), Packham et al., (I978) and Frojmovic (I978). Unless such factors are carefully controlled, spurious results may arise. These technical problems may in part explain the lack of agreement between different clinical studies. For example, increased sensitivity to aggregating agents has been described in patients with stable ischaemic heart disease (O'Brien et al., I966; Goldenfarb et al., I97I; Dreyfuss & Zahavi I973) but not by others (Enticknap et al., I969; Steele et al., I973).

Other tests which measure platelet aggregation in vitro including screen filtration (Swank, I968) and membrane capacitance (Bicher, I97I) do not appear to offer advantages over the conventional aggregometer technique, and have not been widely adopted. Platelet electrophoresis is an interesting though laborious technique. Changes in electrophoretic mobility have been reported in platelets from patients with ischaemic heart disease by Hampton and Mitchell (I974) but others have failed to find this abnormality (Grottum, I968).

In Vivo Tests

Bleeding time

This simple test can be made reasonably reproducible, particularly if the technique modified by Mielke et al. (I969) is used. Lengthening of the bleeding time occurs in certain quantitive and quantitive platelt disorders such as von Willebrand's disease and thrombasthenia, but the technique has not been widely used in thrombotic states (Hirsh et al., I978).

Platelet survival

This approach to the study of platelet behaviour will be discussed in greater detail in Part III, Section I. Though time-consuming, it offers the advantage of assessment of the platelet -vessel wall interaction, rather than of the platelet in isolation.

Platelet aggregate formation

In this technique, blood is taken into anticoagulant (EDTA) with and without formalin. The two samples are centrifuged to sediment any platelet aggregates fixed by the formalin, leaving the remaining platelets in suspension. Platelet counts of formalinized and unformalinized platelet-rich plasma are compared to give an estimate of platelet aggregates (Wu & Hoak 1974). It is not certain if this simple test does really measure circulating platelet aggregates, or whether it reflects in vitro activation of platelets during blood collection. The test appears to be abnormal during acute thrombosis, returning to normal a few days after the acute episode (Dougherty et al., 1977).

Beta-thromboglobulin and platelet factor 4

Plasma measurements of these two proteins have been used extensively as tests of "in vivo" release of platelets in a wide variety of thombotic disorders (reviewed by Zahavi & Kakkar, 1980; Kaplan & Owen, 1981). Both tests readily yield false positives unless blood collection and processing are performed scrupulously to avoid in vitro platelet release. Though a correlation between raised β-thromboglobulin levels and shortened platelet survival has been described (Ludlam et al., 1979; Doyle et al., 1980), neither the administration of sulphinpyrazone (Ludlam et al., 1979; Han et al., 1979) nor aspirin and dipyridamole (Han et al., 1979), both of which prolonged platelet survival, reduced β-thromboglobulin levels, suggesting that plasma levels of β- thromboglobulin and platelet survival do not neccessarily record the same phenomenon.

SECTION 3 THE COAGULATION SYSTEM

THE COAGULATION SYSTEM

HISTORICAL

In I666 Malphigi observed when blood was allowed to clot, and the clot was washed, a mass of white fibres remained (Pickering, I928). Ruysh (I707) discovered that the elastic material obtained by whipping blood was similar to that obtained by washing clots (Pickering, I928) and this material was named fibrin (la fibrine) by Chaptal (I795).

Hewson (I770) showed that coagulation could be inhibited by neutral salts such as sodium sulphate. Arthus and Pagès (I890) established the importance of calcium in fibrin formation and from the work of Hammarsten (I899), Schmidt (I872) and Morawitz (I905) the known clotting factors were named and the so-called "classical" theory of blood coagulation emerged (Fig I).

In essence the theory proposed two separate inactive components (prothrombin and fibrinogen) triggered into a two-stage reaction by an activator (thromboplastin) released by cellular damage, a concept which remained unchallenged for nearly 50 years.

An advance was made possible by the introduction by Quick (I935) of the one-stage prothrombin time test, which was based on the assumption that if calcium, thromboplastin and fibrinogen were present in excess, the clotting time would be a reflection of the prothrombin concentration. In I943 Quick observed that stored human plasma developed a prolonged prothrombin time, which was corrected by the addition of dicoumarized dog plasma. This led Quick to propose that prothrombin consisted of a labile fraction (fraction A) and a stable fraction (fraction B) reduced by dicoumarin. In I947, publishing work performed in I944, Owren described a patient whose plasma had a prolonged prothrombin time which was corrected by addition of a small amount of normal plasma from prothrombin had been removed by adsorption. Owren named the new component factor V, which represented Quick's labile factor. The stable factor was finally designated factor VII by Koller et al. (I95I). The identification of factors V and VII did not greatly alter the classical theory, but a problem arose when it was appreciated that haemophilic patients had a prolonged clotting time in glass tubes, but a normal prothrombin time. Weil (I906) had previously shown that normal blood could correct the long clotting time of haemophilic blood, and

this observation was later confirmed by Pohle and Taylor (1937) and Minot and Taylor (1947) who found that the correction in clotting time was produced by a plasma fraction free of fibrinogen and containing only traces of prothrombin. This factor was termed antihaemophilic factor, and was followed by a series of reports of patients with coagulation abnormalities resulting in the identification of Christmas factor (Biggs et al., 1952; Aggeler et al., 1952), plasma thromboplastin antecedent (Rosenthal et al., 1953), Hageman factor (Ratnoff & Colopy, 1955), Stuart-Prower factor (Telfer et al., 1956; Hougie et al., 1957) and fibrin stabilizing factor (Lorand & Jacobsen, 1958). The confusion over the nomenclature of these factors was resolved by an International Committee who assigned a Roman numeral to each factor (Macfarlane, 1972). More recently, the discovery of two other coagulation factor defects, Fletcher factor (Hathaway et al., 1965) and Fitzgerald factor (Waldman et al., 1975) have provided additional information concerning the initial events in surface-mediated coagulation. A list of the synonyms and their internationally agreed names are shown in Table I.

ACTIVATION OF THE COAGULATION SYSTEM

Blood coagulation is customarily divided into three phases: the intrinsic system, involving activation of factor X following contact with a foreign surface; the extrinsic system, which is the system which is activated when blood is exposed to damaged tissues; and the common pathway which is the sequence of events following activation of factor X to the formation of a fibrin clot.

Activation of the Intrinsic Coagulation System

The intrinsic pathway of coagulation is initiated by surface contact. The contact system of plasma proteins consists of four principal proteins: factors XI and XII, prekallikrein and high-molecular kininogen. Interaction of these substances results not only in the activation of the coagulation system, but also the kinin and fibrinolytic pathways, though the precise mode of the activation process is not yet fully undersood. Probable mechanisms are reviewed more fully by Ogston and Bennett (I978) and Cochrane et al. (I980).

When plasma comes into contact with a negatively-charged surface, the four molecules assemble on the surface, leading to the generation of active enzymes and the conversion of high-molecular kininogen to bradykinin. A number of negatively-charged substances of possible biological importance are capable of inducing contact activation, including collagen (Wilner et al., I968) and vascular basement membrane (Cochrane et al., I972). Once bound to the surface, factor XII is more susceptible to activation by proteolytic cleavage (e.g. by kallikrein; Griffin, I978) and XI activation is enhanced by surface-bound XII and high-molecular kininogen (Cochrane et al., I980). Kallikrein, generated at the surface from prekallikrein by XIIa, in its turn amplifies the reaction by activation of additional factor XII (Cochrane & Revac (I979). Activated factor XII also activates plasminogen (Niewiarowski & Prou-Wartelle, I959).

Factor XIa is a serine protease that converts factor IX to IXa (Davie & Ratnoff, I964). The next zymogen to be activated in the sequence is factor X which also requires calcium ions, phospholipid and factor VIII. The role of factor VIII in factor X activation is not clear, but may serve as a regulatory protein enhancing the rate of factor X activation, in the same way that factor V accelerates the conversion of prothrombin (Esnouf, I977).

Activation of the Extrinsic Coagulation System

The active component of tissue factor is known to be a lipoprotein complex (Nemerson & Pitlick,I972). Tissue factor acts by activation of factor VII, a process which is accelerated by the presence of a number of enzymes such as factor Xa, thrombin, fragments of factor XII and plasmin (Bennett, I977). Factor VIIa, tissue factor and calcium ions result in the activation of factor X (Nemerson & Pitlick, I972).

Activation of the Common Pathway

Activation of factor X by either the intrinsic system or the extrinsic system is achieved by the same mechanism (Jesty et al., I974). Conversion of prothrombin to thrombin requires factor Xa, phospholipid, calcium ions and factor V. This process is reviewed extensively by Esnouf (I977) and Bennett (I977). Activation of factors XII, XI, IX, and possibly VII is accomplished by a common mechanism (Rosenberg, I975). These factors circulate as inactive precursors which are activated when a proteolytic enzyme with serine at its active centre is released by cleavage of bonds. This system of amplification was postulated as the classical cascade or waterfall concept of coagulation in I964 independently by Macfarlane and Davie and Ratnoff and survives with only minor modifications.

The interaction of thrombin with fibrinogen to form fibrin monomer starts the final stage of fibrin formation. Fibrinogen is a glycoprotein of molecular weight approximately 340,000 daltons. Its molecular configuration is still debated (Gaffney, I977; Dolittle, I980). Conversion of fibrinogen to fibrin involves the cleavage of the small polypeptides A and B (Bailey & Bettelheim, I955; Bailey et al., I95I). The resulting soluble protein (fibrin monomer) polymerizes to fibrin polymer, a process which is accelerated by the presence of calcium (Boyer et al., I972). The fibrin clot is rendered more rigid by the formation of γ-glutamyl-ε-lysine links between adjacent fibrin molecules by the action of factor XIII (Lorand & Jacobsen, I958).

INHIBITORS OF COAGULATION

Powerful mechanisms exist in the circulation for limiting fibrin formation. At least four different antithrombins have been described. These include the protease inhibitors α_1-antitrypsin (Gans & Tan, I967), α_2-macroglobulin (Steinbuch et al., I967) and c_1 esterase inhibitor (Ratnoff et al., I969) but the α_2-globulin termed antithrombin III is responsible for the largest part of antithrombin activity in plasma (Lane et al., I975). Antithrombin III, being the major antithrombin in plasma is also the plasma protein through which heparin exerts its effect (Barrowcliffe et al., I978). In addition to its antithrombin properties, in the presence of heparin, antithrombin III inhibits the serine proteases plasmin, the clotting factors XIIa XIa, IXa and particularly Xa (Rosenberg, I975).

Coagulation and Arterial Thrombosis

Since impairment of the coagulation system leads to haemorrhage, it might seem reasonable to search for evidence of hypercoagulability in patients with thrombotic disorders. In general, such a search has proved disappointing, particularly in the case of arterial disease (Davis & McNicol, I978; Hirsh, I98I). Though many studies have attempted to demonstrate an association between raised levels of coagulation factors and arterial disease, the significance of these results may be questioned, since such a relationship may be incidental rather than causal. In contrast to the haemorrhagic disorders, few congenital abnormalities of haemostasis have been shown to result in an increased risk of thrombosis, with the notable exception of antithrombin deficiency. A number of families with hereditary antithrombin III deficiency have been described, with a high prevalence of venous (Egeberg, I965; Marciniak et al., I974) and possibly also arterial disease (Mackie et al., I978).

Though several plasma proteins such as fibrinogen, factor VIII and α_1-antitrypsin rise following tissue injury e.g. myocardial infarction (Brozovic, I977; Hirsh, I98I), these changes are transient, and therefore appear unlikely to provoke further arterial disease, but could favour venous thromboembolism during convalescence.

Longstanding coagulation abnormalities associated with an increased risk of arterial disease are relatively few. Low levels of antithrombin III have been described in patients with stable ischaemic heart disease (Innerfield et al., I976; Yue et al., I976). Fibrinogen concentrations are raised in young patients with ischaemic heart disease (Ogston & Ogston, I966) and more recently, the level of fibrinogen has been shown to correspond to the severity of angiographically-proven coronary artery disease (Lowe et al., I980). However, it cannot be certain that these changes are not merely secondary phenomena, and may be of no pathogenetic significance.

More direct evidence for direct involvement of the coagulation system in arterial thrombosis has recently been published. Preliminary results of the Northwick Park prospective study of cardiovascular disease in the community (Meade et al., I980) has shown that the mean recruitment levels of factors VII, VIII coagulant activity and fibrinogen were significantly higher in those who died of cardiovascular disease compared to those who survived.

SECTION 4 THE FIBRINOLYTIC SYSTEM

THE FIBRINOLYTIC SYSTEM

HISTORICAL

Mammalian blood contains an enzyme system capable of dissolving blood clots - the fibrinolytic system. Like the coagulation system, a complex arrangement of inter-related pathways have been described, though the precise physiological and pathological significance of the process is not fully understood.

Morgagni (I769) and Hunter (I793) were aware that blood remained fluid in the blood vessels after sudden or violent death. Green (I887) discovered that fibrin formed from ox blood dissolved after incubation with saline, and that the resulting solution could not be clotted by thrombin. The term fibrinolysis (la fibrinolyse) was coined by Dastre (I893) and Hedin (I903) demonstrated that the ability to lyse clots resided in the globulin fraction of serum. Milstone (I94I) showed that a substance produced by streptococci required a factor present in human serum to lyse blood clots. This lytic factor was studied by Kaplan (I944) and Christensen (I945) who concluded that it was an enzyme precursor which was activated by the streptococcal fluid. Subsequently, this precursor was named plasminogen, the enzyme plasmin and the streptococcal factor streptokinase (Christensen & Macleod, I945). These historical aspects have been reviewed more extensively by Astrup (I956) and McNicol and Douglas (I972).

COMPONENTS OF THE FIBRINOLYTIC SYSTEM

Plasminogen

Human plasminogen is a single chain glycoprotein, with a molecular weight of approximately 90,000 daltons (Soktrup et al., I978). Native plasminogen has an NH_2-terminal glutamic acid (Glu-plasminogen) but it is readily converted by plasmin digestion to modified forms with NH_2-terminal lysine, valine or methionine (Wallén & Wiman, I970, Wallén & Wiman, I972). The biological significance of these two forms is uncertain, but it seems that virtually all the normal circulating plasminogen in man is in the Glu-plasminogen form (Collen et al., I975). Plasminogen contains an arginyl-valine bond which is cleaved by plasminogen activators to form plasmin (Robbins et al., I967).

Plasminogen Activators

Plasminogen activators occur in the blood and in many different tissues and body fluids such urine, saliva and semen (Astrup, I966, McNicol & Douglas, I972). A small amount of plasminogen activity can be detected in normal blood (Müllertz, I953), but levels rise following various stimuli such as strenuous exercise, stress or venous occlusion (Brozovic, I977). This activity originates mainly from the vessel wall (Todd, I964). Recent studies have shown that cultured vascular endothelial cells are capable of synthesizing plasminogen activator (Levin & Loskutoff, I979) though inhibitors of activation are also produced. Loskutoff (I979) has shown that when endothelial cells are stimulated with thrombin, plasminogen activator is markedly reduced, which may have important implications during thrombus formation in vivo.

Plasminogen activator activity can also be induced by contact activation of factor XII (Niewiarowski & Prou-Wartelle, I959). This so-called "intrinsic" fibrinolysis, to distinguish it from plasminogen activation by tissue or vascular activator ("extrinsic" fibrinolysis; Collen, I980) requires the presence of other proteins, one of which has been termed Hageman factor cofactor (Ogston et al., I969). Various pathways involving factor XII, high molecular kininogen, prekallikrein and contact activation have been proposed (Kaplan et al., I976, Ogston & Bennett, I978), though the precise mechanisms and physiological importance of intrinsic plasminogen activation is unknown.

Inhibitors of Fibrinolysis

Inhibitors of plasminogen activation

Inhibitors of intrinsic plasminogen activation include c_I inactivator (Kluft, I977), an inhibitor of XIIa-induced fibrinolysis (Hedner & Martisson, I978) and α_2-macroglobulin (McConnell, I972). Since the physiological role of the intrinsic pathway of activation is unknown, the importance of these inhibitors in vivo remains conjectural. Inhibitors of the extrinsic pathway have also been described, but their existance has not been definitely established (Collen, I980).

Inhibitors of plasmin

Antiplasmins have been more fully characterized than inhib-

itors of plasminogen activation. The principal physiological inbibitor of plasmin appears to be α_2-antiplasmin (Collen, I976, Müllertz & Clemmensen, I976). A number of other substances possess antiplasmin activity including α_2-macroglobulin (Ganrot, I967), α_I-antitrypsin (Rimon et al., I966), antithrombin III (Highsmith & Rosenberg, I974) and c_I esterase inhibitor (Ratnoff et al., I979).

Mechanisms of Fibrinolysis

The mechanisms of fibrinolysis in vivo remain controversial, and a number of hypotheses have been proposed to explain how fibrinolysis is localized to the fibrin clot. Alkjaersig et al. (I959) have suggested that plasminogen is adsorbed to polymerizing fibrin and converted to an active enzyme by activators which diffuse into the clot. In this environment, plasmin could act relatively free of inhibitors. Ambrus and Markus (I960) proposed that plasmin-inhibitor complexes, formed in the circulation dissociate in the presence of fibrin, since plasmin has a higher affinity for fibrin than for its inhibitors. Chesterman et al. (I972) have advanced the hypothesis that the activators bind selectively to fibrin and convert plasminogen, which diffuses into the clot, to plasmin. A more recent hypothesis, based on a molecular model, has been formulated by Collen (I980). He has proposed that plasminogen binds to fibrin by its lysine-binding sites. Tissue plasminogen activator also binds strongly to fibrin, thereby activating the fibrin-bound plasminogen. Since the fibrin-bound plasmin which results has a relatively short half-life (I0 s), effective clot lysis depends on a continuous replacement at the clot surface of inactivated plasmin molecules (complexed with α_I-antiplasmin) by plasminogen molecules.

Whatever mechanism is responsible for clot and thrombus dissolution in vivo, many other factors may be important such as age of thrombus, site (arterial or venous) presence of blood cells and the extent of fibrin cross-linking (Kernoff & McNicol, I977). The existence of other plasmin-independent fibrinolytic mechanisms has also been described, though their importance in vivo is uncertain (Plow & Edgington, I975).

Degradation of Fibrin

When fibrin is exposed to plasmin, digestion occurs continuously over long periods, forming a variety of degradation products which vary in size and properties (Marder et al., I969; Marder & Schulman, I969). These products are referred to collectively as fibrin degradation products (FDP), fibrinolytic split products (FSP) or fibrin-fibrinogen related antigen (FR-antigen). Fibrin is initially split to yield fragment X (MW 270,000) and low molecular fragments A, B and C. Fragment X is split to fragment Y (MW I65,000) and fragment D (MW 85,000). Finally, fragment Y is digested to fragments D (MW 85,000) and E (MW 55,000), (Marder et al., I969; Marder & Schulman, I969). Fragment X is slowly clottable by thrombin, but the final products of plasmin digestion are non-clottable (Pizzo et al., I972).

Fibrinolysis and Thrombotic Disease

Some authors have proposed that fibrinolysis is in dynamic equilibrium with blood clotting, and that in health fibrin deposition and lysis occurs continuously as a part of the normal maintenance of blood vessels (Nolf, I908; Astrup, I956). In support of this view is the observation that plasminogen activator activity is present in non-stressed blood (Müllertz, I953). However, turnover studies with radiolabelled fibrinogen and plasminogen show that low grade intravascular coagulation and/or fibrinolysis is very low or non-existent (Collen et al., I972; Harker & Slichter, I974).

Though a partial deficiency of plasminogen associated with recurrent thrombosis has been described (Aoki et al., I978), other patients have low or absent levels of functional plasminogen without thrombotic problems (Jacobsen, I968; Aoki et al., I978) suggesting that non-plasmin dependent fibrinolytic pathways may be important. Plasminogen levels are not generally reduced in arterial thrombotic diseases (Ogston & Ogston, I966; Harker & Slichter, I974). Though plasminogen turnover is increased in patients with venous thrombosis, it is unaltered in arterial thromboembolic disease (Harker & Slichter, I974). A few patients with a deficiency of inactivators of plasminogen activator (Nilsson et al., I96I; Pandolfi et al., I970) and reduction in antiplasmin (Naeye, I96I) associated with severe thrombotic disease have been described, but such cases seem to be very rare.

Apart from these uncommon deficiency states, the evidence for impaired fibrinolysis as a contributing factor to thrombosis is less convincing. Reduced fibrinolytic activity, measured by a variety of techniques such as spontaneous activity, stimulation by venous occlusion and quantity released by vein wall biopsies, has been reported in a variety of conditions including coronary artery disease (Chakrabarti et al., I968), peripheral vascular disease (Nestel, I959), cerebral vascular disease (Pilgeram, I974) and oral contraceptive usage (Astedt et al., I973). However, the significance of these studies may be questioned, since the reduced fibrinolytic activity may have been secondary to the thrombotic condition. Recently, two prospective studies suggest that reduced fibrinolytic activity may identify which patients are at risk of thrombotic disease. Reduced fibrinolytic activity was found to be the most powerful predictor of deep vein thrombosis in women undergoing gynaecological surgery (Clayton et al., I976) and preliminary results from the Northwick Park prospective study of cardiovascular death suggested that fibrinolytic activity at the onset of the study tended to be lower in subjects dying from cardiovascular causes compared with the survivors, though this difference did not achieve statistical significance (Meade et al., I980).

PART II THE HAEMOSTATIC SYSTEM IN DIABETES MELLITUS

THE HAEMOSTATIC SYSTEM IN DIABETES MELLITUS

This initial study was designed to assess the extent of platelet, coagulation and fibrinolytic system abnormalities in a representative sample of ambulant patients attending a diabetic clinic in comparison with a non-diabetic control group. The battery of tests was selected to give the maximum information on the haemostatic system, with a balance between proceedures performed on the morning of venesection and others which could be performed on frozen plasma samples.

For ease of presentation, the sections concerning platelet aggregation, tests of coagulation and tests of fibrinolysis will be considered separately.

SECTION I PLATELET AGGREGATION AND DIABETES MELLITUS

INTRODUCTION

A wide range of abnormalities of platelet behaviour have been described in diabetic patients and have been extensively reviewed by Bern (I978), Colwell et al. (I978) and Jones and Peterson (I98I). This section concerns platelet aggregation; other aspects of platelet function in diabetes will be discussed in PartIII, Sections I and 2 and in Part V, Section 2.

Aggregation in vitro is perhaps the most intensively studied aspect of platelet behaviour in diabetes. The most commonly used aggregating agent used has been ADP, though aggregation to adrenaline, collagen and arachidonic acid have also been studied. The earliest studies used a system whereby aggregation of recalcified PRP was observed visually in a rotating Chandler's loop or disc. Szirtes (I970) and Rathbone et al. (I970) found that the time taken to produce visible platelet aggregates was shorter in diabetic subjects than controls, and that this abnormality was most marked in patients with vascular disease. These results could be interpreted as evidence of increased thrombin generation in the PRP from diabetics rather than hyperaggregability of platelets per se, but subsequent reports of abnormal aggregation measured using the platelet aggregometer (a system independent of the coagulation system) began to appear. To date, results have been somewhat contradictory. The majority of authors have reported that diabetics with evidence of severe microvascular disease have an abnormally low threshold or an increased extent of the second phase of aggregation induced by ADP (Heath et al.,I97I; Kwaan et al., I972a; Leone et al., I974; Passa et al., I974; Bensoussan et al., I975; O'Malley et al., I975) in both insulin dependent and non-insulin dependent patients. The group of Colwell have been able to demonstrate enhanced second phase aggregation to ADP and adrenaline in 'latent' diabetics, defined as asymptomatic individuals with abnormal glucose tolerance (Sagel et al., I975; Colwell et al., I976; Colwell et al., I977) but in contrast, Davis et al. (I974) were unable to demonstrate any differences in the extent of ADP induced aggegation between men with and without abnormal glucose tolerance. Recently, Petersen and Gormsen (I978) were unable to demonstrate any differences between ADP induced aggregation of insulin dependent diabetics with and without retinopathy and non diabetic controls.

In general, these studies have involved groups of patients with a high prevalence of severe microvascular complications. The present study was designed to assess platelet aggregation in a representative sample of diabetics attending a diabetic clinic, with particular emphasis on macrovascular complications.

MATERIALS AND METHODS

SUBJECTS

Thirty-four diabetics (20 men, I4 women) were chosen from those patients attending the Aberdeen Diabetic Out-Patient Clinic. Selection criteria included abnormal glucose tolerance (see below) and a willingness to participate in the study (which frequently involved loss of a morning's work).No age criteria were imposed, though patients taking regularly drugs other than insulin or oral hypoglycaemic agents and pregnant diabetics were excluded from the study.

Six patients were chemical diabetics (defined as asymptomatic individuals with an Increment Index of more than 2.46 in response to an intravenous glucose tolerance test (Duncan, I956). The remainder were clinical diabetics as defined by Fitzgerald and Keen (I964). Eighteen were treated with insulin, I4 with oral hypoglycaemic agents and two were controlled on diet alone.

Thirty-four non-diabetic subjects (22 men, I2 women) were selected as controls from laboratory and medical staff of Aberdeen Royal Infirmary and staff of the Macaulay Institute for Soil Research, Aberdeen. All controls had a fasting plasma glucose level of less than 6.I mmol/l and none were receiving regular medication.

Subjects were asked to abstain from all drugs apart fomm diabetic treatment for at least I4 days prior to the test. Informed consent was obtained from all taking part in the study.

After an overnight fast, subjects attended the ward. Diabetics postponed their morning tablets or insulin until the test was completed. While resting supine, a history and examination was undertaken. Specific enquiries were made to elicit a past or present history of angina, dyspnoea, myocardial infarction, claudication, stroke, hypertension, vascular surgery and smoking. Blood pressure was measured and peripheral pulses were palpated. A search for femoral, carotid and vertebral bruits was made and a I2-lead electrocardiograph (ECG) was performed. The retinae were inspected by direct ophthalmoscopy (pupils undilated) and finally the subjects' height and weight were measured.

A diagnosis of ischaemic heart disease (IHD) was based on a history of myocardial infarction confirmed by ECG or a rise in

cardiac enzymes, cardiac failure or angina or ischaemic changes on the ECG (Q waves, T inversion, S-T depression >2 mm); peripheral vascular disease (PVD) as a history of arterial surgery of the lower limbs, intermittent claudication, presence of femoral bruits or absence of more than two leg pulses; and cerebrovascular disease (CVD) as a history of stroke of presumed thromboembolic origin, transient ischaemic attacks or presence of carotid or vertebral bruits. An obesity index was calculated by dividing the subject's weight by an ideal weight standardized for height, sex and age from tables compiled by Kemsley et al. (1962).

After 15 min resting supine, 45ml blood was removed from an antecubital vein, using minimal venous stasis and a 19 gauge needle.

REAGENTS

Adenosine 5' Diphosphate Grade I Sodium Salt (Sigma Chemical Co., St. Louis, Missouri) was dissolved in barbital-saline buffer, pH 7.35 (Sagel et al., 1975) to give a 2.1 mM stock solution which was aliquotted into polythene containers and frozen at -20°C until use. After thawing, any unused solution was discarded. The stock solution was renewed every three months. Fresh dilutions of the stock were prepared daily.

Epinephrine (Sigma Chemical Co.) was dissolved in 0.1M hydrochloric acid to give a stock solution of 10.5mM which was aliquotted and frozen until use at -20°C. The stock was renewed weekly. Fresh dilutions of the stock were made up in barbital-saline buffer, pH 7.35, each day.

Collagen Fibrils (Hormon-Chemie, Munich, W. Germany) were stored in suspension at 4°C and dilutions were made daily, using glycine buffer, pH 2.8.

METHODS

Platelet Count was performed according to the method of Brecher and Cronkite (1950). 0.1ml of the sample (either anticoagulated blood or platelet-rich plasma (PRP)) was pipetted into a plastic tube containing 1.9ml of a freshly prepared 1% aqueous solution of ammonium oxalate. The contents of the tube were gently mixed, and when whole blood was used, left to stand for 10min to allow haemolysis of the erythrocytes

to occur. A small volume of the well mixed platelet suspension was transferred to both chambers of an improved Neubauer counting chamber (Hawksley & Sons Ltd., Lancing, Sussex). The chamber was left in a damp Petri dish for a minimum of 20 min to allow the platelets to settle. The platelets were then counted using phase contrast microscopy. A minimum of 200 platelets were counted on each side of the chamber and the average of the two counts (x 100) was taken as the platelet count /mm^3. When the count in the two sides differed by more than 1%, the counts were repeated using a fresh chamber.

Platelet Aggregation Venous blood was anticoagulated with 1.29M trisodium citrate in a ratio of 1 : 19 to give a final concentration of 0.064mmol/l. The citrated blood was centrifuged at 250 g for 10 min. The resulting PRP was removed using a plastic pipette and placed in an unstoppered polypropylene tube. Care was taken to wipe the sides of the tube after centrifugation, to avoid erythrocyte contamination of the PRP. The buffy coat was left undisturbed and the remaining red cells and plasma were centrifuged at 1,700 g for a further 10 min to obtain platelet-poor plasma (PPP), which was pipetted into a clean tube. 0.1ml PRP was removed for platelet count and the remainder was transferred into disposable acrylic cuvettes (ADG Instruments Ltd., Crawley, Surrey) with a capacity of 0.2ml, using an automatic pipette with disposable polypropylene tips, 0.2ml PPP being placed in a similar cuvette. To each was added a 3mm x 1mm disposable siliconized stir bar (ADG Instruments Ltd.). Centrifugation and storage of the samples were at room temperature.

The twin-channel platelet aggregometer (ADG Instruments Ltd.) was connected to a twin-channel chart recorder (Smith Instruments Ltd., Wembley). Fifteen min before the start of the experiment, the aggregometer was switched on to warm the heating block to 37°C, and the stirring speed was set at 1,200 rpm . The optical transmission of PRP was arbitrarily set at 0% and that of PPP at 100%. Since the light transmission of plasma from diabetic subjects varied owing to lipaemia, the instrument settings required adjustment for each subject studied, but were not altered during a single run. Chart speed was set at 10mm/min.

On a single day, only one subject was studied (diabetic or control). In parallel with the subject's aggregation response, to act as a control for the aggregating agents, a second sample of PRP

was tested in the second channel. This PRP was provided by one of two non-diabetic subjects, the same two donors being used thoughout the experiment.

Three min before aggregation, a sample of PRP was incubated at 37°C in a water bath. The cuvette was removed after exactly 3min, the sides dried, and placed in the aggregometer. The aggregating agent was added using a Terumo Micro Syringe (25ul capacity, Glass Appliances Ltd., Aberdeen), taking care to avoid air bubbles. Aggregation was followed for 3 min. Serially increasing concentrations of each agent were added until a biphasic response (ADP, adrenaline) or a monophasic response (collagen) was obtained up to 3 min after addition of the aggregating agent (Figs. 2, 3, 4). The sensitivity of the platelets to each aggregating agent was defined as the threshold concentration required to produce a biphasic (ADP, adrenaline) or monophasic (collagen) response within 3 min of addition of the aggregating agent (O'Malley et al., 1975).

Determination of the threshold ADP concentration was started precisely 20 min after venepuncture and was completed within 20 min. Between 40 and 60 min the threshold for collagen was determined, followed by adrenaline between 60 and 80 min. The time taken to complete the experiment never exceeded 80 min from venesection.

Statistics To compare results obtained in diabetics and controls, in view of the near normal distribution of the threshold concentrations and platelet counts, Student's t test for non-paired data was employed.

In view of the smaller numbers involved, Wilcoxon's rank sign test for non-paired data was used to compare the results obtained from different diabetic sub-groups.

RESULTS

CLINICAL DATA (Tables 2 and 3)

Age The mean age of the diabetics was 47.I years (SD ± I6.7) which was significantly older than that of the controls (39.I ± I0.7 years; $p < 0.025$). The age distributions are shown in Fig. 5.

Obesity The mean obesity index of the diabetics was II2.4 ± I5.4, which was not significantly different from that of the controls (I07 ± I0.7; $p < 0.05$). The distributions of obesity index are shown in Fig. 5.

Blood Pressure The mean systolic blood pressure of the diabetics was significantly ($p < 0.025$) higher than that of the controls (I42 ± 25 compared to I29 ± 20 mmHg), but the mean diastolic pressures were not significantly different (84 ± I2 compared to 82 ± II mmHg; $p > 0.05$). As can be seen from Fig. 6, only two diabetics and one control had diastolic blood pressures above I00 mmHg.

Smoking Eight diabetics and six controls were regular cigarette smokers.

Vascular Disease None of the controls had clinical evidence of atherosclerotic vascular disease. The prevalence of macrovascular disease of the diabetics is shown in Table 2. Fourteen of the 34 diabetics had clinically evident large vessel disease. Only two diabetics had severe microvascular disease, one with proliferative retinopathy, and one with a severe peripheral neuropathy. No diabetic had proteinuria detectable by test strips (Labstix, Ames Co.). Since fluorescein angiography was not performed on these patients, and ophthalmological examination was carried out with the patients' pupils undilated, no separation was made between absence of retinopathy and minimal background changes.

PLATELET TESTS

The results of platelet counts and platelet aggregation are summarized in Table 4.

Whole Blood Count There was no significant differences in mean platelet counts between diabetics and controls. Similarly, there were no differences between diabetics with and without vascular disease, or between insulin dependent and non-insulin dependent diabetics.

PRP Platelet Count The mean platelet count of PRP was slightly, but significantly ($p < 0.05$) lower than that of the controls, but there were no significant differences between diabetics with and without vascular complications and between those on insulin and those on oral therapy.

ADP Threshold From Fig. 7 it can be seen that there was no difference in the threshold concentration of ADP required to produce a biphasic response in diabetics and controls, or between the different diabetic sub groups.

Adrenaline Threshold As shown in Fig. 8, the threshold concentration of adrenaline required to produce a biphasic response was similar in diabetics and controls, and in the diabetic sub groups.

Collagen Threshold The threshold concentration of collagen required to produce platelet aggregation was similar in diabetics and non-diabetics, with no significant differences between those with and without vascular disease, and between those on insulin and those on oral therapy (Fig 9).

In diabetic subjects Nos. I2, I5 and I7 no second phase response was obtained to ADP and adrenaline and their platelets did not aggregate in response to collagen. Though subjects had been specifically requested to refrain from taking aspirin-containing drugs, this would appear to be the most likely explanation for the lack of response. In four diabetics and four controls it was not possible to determine the threshold for collagen, in four controls the threshold for adrenaline and in three controls the threshold for ADP could not be determined within the allotted 20 min timespan.

Aggregation Control The thresholds for a single normal donor were measured 60 times over the time of the study, in parallel with the diabetic and non-diabetic subjects. Mean $\pm$ SD threshold concentration for ADP was 0.9I $\pm$ 0.22 uM (range 0.6-I.6), for adrenaline 0.29 $\pm$ 0.I5 uM (range 0.I-0.7) and for collagen 0.37 $\pm$ 0.I3 ug/ml (range 0.2-0.7), giving coefficients of variations of 24.2, 5I.7 and 35.6% respectively.

DISCUSSION

This study was unable to show significant differences in the threshold concentrations of ADP, adrenaline and collagen required to produce primary (collagen) or secondary (ADP and adrenaline) platelet aggregation in a group of ambulant diabetic out-patients compared to non-diabetic controls. The diabetic group were significantly older than the controls, which might have been expected to exaggerate any differences between the two groups (Lecrubier et al., I980). The diabetics had a significantly higher systolic blood pressure, but were otherwise well-matched for diastolic blood pressure, obesity and cigarette smoking.

In spite of careful standardization of conditions of rest, nutrition, temperature of centrifugation and aggregation, and timing of venesection and platelet aggregation, using the same non-diabetic control on 60 occasions, the coefficient of variation for ADP, adrenaline and collagen was wide. Similar day-to-day variability has been reported by others (Harrison et al., I967; Goldenfarb et al., I97I). The cause of this variability is not known, but could include minor differences in the shape of stir bars or cuvettes (Coller & Gralnick, I976) or ill-defined factors such as mental stress (Harrison et al., I967).

The mean platelet count of PRP was significantly lower in diabetics than in controls, though the platelet counts in whole blood were similar. This difference has not been reported previously. Blood from diabetic subjects appears to contain a higher proportion of megathrombocytes than non-diabetics (Garg et al., I972; Colwell et al., I977) and it is possible that during centrifugation some of these denser platelets may have been discarded in the buffy coat. A second possibility is that sedimentation of platelets from diabetics was accelerated by the higher concentration of plasma fibrinogen in these subjects (see Part II, Section 2). The effect of fibrinogen on platelet sedimentation is not known, though this plasma component appears to be the most important determinant of erythrocyte sedimentation under normal conditions (Zacharski, I976). Whatever the reason, the lower platelet count of PRP from diabetic subjects may have obscured any difference between the groups, since platelet aggregability increases with increasing platelet count (Born & Hume, I967; O'Brien, I97I).

In the present study, the platelet counts of PRP were not adjusted to a constant number since it was decided to attempt to complete aggregation studies within 80 min of venesection to minimize the known changes in platelet aggregability with time 'temporal drift' (Hardisty et al., I970; Warlow et al., I974; Rossi & Louis, I975). This time limit precluded the determination and adjustment of platelet count by visual methods. However, the difference in mean platelet counts between diabetics and controls was small (approximately 70 x 10^9/l) and when the platelet count of the PRP of the 60 control ADP-induced aggregations using the same donor were correlated with the threshold concentration of ADP, only a weak correlation was found ($r = 0.27$; $p > 0.05$). This suggests that variation in platelet count was not the only factor causing the relatively large coefficients in variation. It follows that any abnormality of platelet aggregation in the diabetic group would have to be considerable to obtain statistically different results from the controls.

In view of the essentially negative findings in this part of the study, the technique of platelet aggregation used in Part III, Section 2 was considerably modified to include standardized PRP platelet counts and a kinetic analysis of the data in an attempt to increase the sensitivity of the method.

SECTION II THE COAGULATION SYSTEM AND DIABETES MELLITUS

INTRODUCTION

In comparison with the intense interest aroused by platelets and the fibrinolytic system in diabetes, the coagulation system has attracted less attention. Early studies described abnormalities of various global tests of coagulation including shortened whole blood clotting, recalcification and thromboplastin times, and increased prothrombin and antiheparin activities, suggesting that diabetic blood may be in a hypercoagulable state (Egeberg, I963; Valdorf-Hansen, I967; Ghanen et al., I97I).

A number of investigators have shown that plasma fibrinogen concentrations are raised in diabetics, particularly in those patients with severe retinopathy or nephropathy (Egeberg, I963; Mayne et al., I970; Almer & Nilsson, I975). However, there is less agreement concerning the levels of the other coagulation factors. Factor VIII:C levels have been reported to be increased (Egeberg et al.,I963; Mayne et al., I970) though others have failed to confirm these findings (Bensoussan et al., I975). Abnormalities of factor VIII (VIII:C and VIII:vWF) will be discussed more fully in Part V , Sections I and 2.

Egeberg (I963) reported raised levels of factor V, but normal levels of factors II, VII, IX, XI and XII whereas Valdorf-Hansen found increased concentrations of factor VII in diabetics with nephropathy and severe retinopathy.

The majority of these studies have been fragmentary. The present study was designed to measure a wide range of coagulation factors, and to relate any abnormalities to the presence of macrovascular complications and to the type of treatment received by the patients.

MATERIALS AND METHODS

SUBJECTS

Diabetics and controls were those already described in the preceeding Section.

REAGENTS

Human Fibrinogen (Kabi Grade L, AB Kabi, Stockholm, Sweden) was dissolved in distilled water to give a concentration of I0g/l and stored in aliquots at - 20°C until use.

Fibreglass (Tyglass fabric quality No. Y83, Fothergill & Harvey Ltd., Manchester) was cut into pieces approximately 30cm x 30cm. The pieces were heated without boiling in a mixture containing three parts concentrated sulphuric acid to one part concentrated nitric acid for I0 min to remove the fabric plasticizer. The squares were washed free of acid with distilled water, air dried and cut into 4cm x 4cm squares.

Folin-Ciocalteu's Phenol Reagent (BDH Chemicals Ltd., Poole, Dorset) was purchased every two months to ensure fresh reagent and stored in the dark until use.

Bovine Thrombin (Parke Davis, Detroit, Michigan) was reconstituted with the addition of the supplied diluent to give a final concentration of 50 NIHu/ml.Aliquots were stored at - 20°C until use.

Platelet Substitute (Platelin, General Diagnostics, New Jersey) was reconstituted to maker's instructions with distilled water.

Barbital Saline Buffer 2.06g barbitone sodium, 2.76g barbitone and 7.3g sodium chloride were dissolved in distilled water and the pH adjusted to 7.5 with IN hydrochloric acid. The volume was made up to Il with distilled water.

BLOOD SAMPLES

Plasma Pool 20ml venous blood was removed from each of 20 healthy male volunteers aged 20 - 30 years. The blood was placed in tubes containing trisodium citrate to give a final concentration of 0.I29M. The tubes were mixed and chilled on ice for 5 min then centrifuged at I,000 g for I0 min. The resulting plasma was pooled in a chilled plastic

beaker and gently mixed. Aliquots were stored at - 70°C until use. Once thawed, any unused plasma was discarded.

Citrated Plasma (0.I29M) was used for the estimations of prothrombin times, partial thromboplastin times and coagulation factor assays. After venesection, the blood was cooled for 5 min before centrifugation and the plasma was stored in small aliquots at - 20°C (partial thromboplastin time, factors VII, X, XI, XII) and at - 70°C (factors II, V, VIII, IX).

Oxalated Plasma was used for measurement of fibrinogen by adding blood to a tube containing dry potassium oxalate in a concentration of 2mg/ml blood. Thereafter the anticoagulated blood was treated in the same way as the citrated blood, and stored at - 20°C.

ASSAYS OF THE COAGULATION SYSTEM

Whole Blood Clotting Time was based on the method of Lee and White (I9I3). One ml venous blood was placed in a triplicate set of polypropylene tubes. The tubes were incubated at 37°C and one tube was tilted every 30 s until clotting occurred. The second tube and then the third tube were treated in the same way until clotting occurred, the clotting time being taken as that of the third tube.

Partial Thromboplastin Time (PTT) was based on the method of Langdell et al. (I953). 0.5ml platelet substitute (reconstituted)was added to 2ml kaolin suspension (5mg/dl) and the mixture was kept on ice. 0.Iml of this mixture was added to a glass tube with 0.Iml test plasma, warmed at 37°C for 60 s and then 0.025M calcium chloride was added. The tube was gently tilted until clotting occurred. The average time in seconds for the plasma to clot after addition of calcium chloride in duplicate tubes was taken as the PTT. A PTT ratio was obtained by dividing the test PTT by a PTT using the normal plasma pool.

Prothrombin Time (PT) was a modification of the method of Quick(I935). A saline extract of brain thromboppastin was thawed and kept on ice. 0.Iml test plasma was mixed with the same volume of brain thromboplastin. After incubation at 37°C for 60 s , calcium chloride (0.I ml of 0.025M) was added. The tube was tilted until clotting occurred. PT and PT ratios were expressed in the same way as for the PTT.

Factor VIII and IX assays were based on the method of Breckenridge and Ratnoff (1962). 0.1ml kaolin/platelet substitute and 0.1ml factor deficient plasma were incubated at 37°C for 8 min (factor deficient plasmas provided by General Diagnostics, Thame, Oxon). The test plasma was diluted 1 : 20 with barbital-saline buffer, pH 7.5. After 8 min the diluted test plasma was added to the tube in the water-bath and mixed. 0.1ml 0.025M calcium chloride was added and the clotting time recorded. In order to obtain a standard curve, dilutions of the normal plasma pool (1 :10, 1 : 20, 1 : 40, 1 : 80) were made and clotting times were established as described above. The standard curve was drawn on double log paper and the test plasma was read off the curve (expressed as per cent normal pool).

Factors XI and XII were assayed by a similar method to that described for factors VIII and IX with the following modifications : kaolin/ platelet substitute mixture and the diluted test plasma were incubated for 2 min at 37°C when 0.1ml factor deficient plasma was added and the tube left for a further 6 min. Calcium chloride was then added and the clotting time recorded.

Factor X was measured using the method described by Denson (1961). 0.1ml factor deficient plasma was mixed with 0.1ml diluted test plasma (1 : 20). 0.1ml reconstituted Russell's viper venom/cephalin reagent (General Diagnostics) was added, the tube mixed and after 30 s 0.1 ml calcium chloride was added, the time taken to clot being recorded.

Factors VII, V and II were assayed using a modification of the method described by Denson (1966). 0.1 ml factor deficient plasma was added to a glass tube and 0.1 ml test plasma (diluted 1 : 20) was added and after 30 s calcium thromboplastin (Behringwerk AG, Marburg-Lahn, W. Germany) was added. The time taken to clot was measured.

Fibrinogen was measured using a modification (Ogston & Ogston, 1966) of the method of Ratnoff and Menzie (1951). To 0.25ml oxalated plasma was added 4.5ml 0.15M saline in a plastic tube. 0.2ml bovine thrombin (50 NIHu/ml) was added and the plasma allowed to clot for 15 min at 37°C. The clot was filtered through the fibre glass square, rinsed with saline, dried and boiled for 10min in 0.2N sodium hydroxide. The solution was cooled and 6ml 12.5% aqueous solution of sodium carbonate followed by 1ml Folin Ciocalteu's reagent (diluted 1 : 2 with distilled water) were added and mixed thoroughly. The solution

was left for I5 min and then centrifuged for 5 min at I000 g before reading in a spectrophotometer at a wavelength of 690nm. The optical density of the solution was read and the concentration of fibrinogen read from a standard curve using purified human fibrinogen.

STATISTICS

Results between diabetics and controls were compared using Student's t test for unpaired samples. In view of the smaller numbers involved and the non-parametric nature of some of the data, comparisons between diabetic sub groups were made using the Wilcoxon rank sign test for non-paired data. Correlations were calculated using linear regression, the results being expressed as the correlation coefficient r.

RESULTS

The results of the coagulation studies are summarized in Table 5. The distribution of the individual coagulation factors which were significantly different in diabetics and controls are shown in Fig. IO.

WHOLE BLOOD CLOTTING TIME

There was no significant difference between diabetics and controls, between diabetics with and without vascular disease and between insulin and non-insulin diabetics.

PARTIAL THROMBOPLASTIN TIME

There was no significant difference between diabetics and controls, or between the different diabetic sub groups.

FIBRINOGEN

Fibrinogen concentrations were significantly higher in diabetics compared with controls ($p < 0.005$). However, there were no statistically significant differences between the diabetic sub groups. Fibrinogen concentrations did not correlate significantly with age, duration of diabetes or obesity index (Table 6).

FACTOR II

Levels of factor II (prothrombin) were significantly higher in diabetics ($p < 0.00I$), but there were no statistically significant differences between diabetic sub groups and factor II levels did not correlate significantly with age,duration of diabetes or obesity index (Table 6).

FACTOR V

Diabetics had significantly higher levels of factor V than controls ($p < 0.00I$). There were no statistically significant differences between diabetic sub groups, and there was no significant correlation with age, duration of diabetes or obesity index (Table 6).

FACTOR VIII

VIII:C activity was significantly higher in diabetics than controls ($p < 0.02$) though there were no significant differences between the different diabetic sub groups, and no significant correlation was found in relation to age, duration of diabetes or obesity index.

FACTOR IX

There was no significant difference between diabetics and controls, or between different diabetic sub groups.

FACTOR X

There was no significant difference between diabetics and controls or between different diabetic sub groups.

FACTOR XI

Factor XI levels were significantly lower in diabetics than controls ($p < 0.001$), but there were no significant differences between diabetic sub groups. There was no significant correlation between factor XI levels and age, duration of diabetes or obesity index (Table 6).

FACTOR XII

There was no significant difference between diabetics and controls, or between different diabetic sub groups.

DISCUSSION

This study has shown significantly increased levels of fibrinogen and factors II, V, VII and VIII in diabetic patients compared with controls. Levels of factor XI were found to be significantly lower in diabetics than controls, whereas there were no significant differences in PTT ratio, whole blood clotting time and levels of factors IX,X and XII. The abnormalities were not statistically significantly related to the presence or absence of macrovascular disease, type of treatment, obesity, age or duration of diabetes. However there was a tendency for levels of fibrinogen and factor VII to be highest in patients with evidence of large vessel disease, though these differences failed to reach statistical significance. It is possible that with larger numbers of patients would produce significant differences.

One possible explanation for the raised coagulation factor levels is that the diabetics were significantly older than the controls, and levels of factors V and VII tend to increase with age in normal subjects (Hamilton et al., I974a). However, no significant correlation between any of the clotting factor levels and age of the diabetics was found, making this explanation less likely.

An unexpected finding was the significantly lower levels of factor XI in diabetics compared with controls. From Table 5 it can be seen that factor XII levels were appreciably lower in both diabetics and controls. These findings may reflect activation of the intrinsic coagulation system followed by inactivation during blood collection and it may be that contact activation of diabetic plasma occurs more readily than control plasma.

This study confirms earlier reports that fibrinogen concentrations of fibrinogen are increased in diabetics (Egeberg, I963; Mayne et al., I970; Almer & Nilsson, I975). More recently, in a large population study, Fuller et al. (I979) found that fibrinogen concentrations were related to the presence of retinopathy. On the other hand, de Silva et al; (I979) in another large study found no relation with the presence of micro- or macrovascular complications, but fibrinogen concentrations appeared to be increased in patients who were receiving sulphonylureas. The results of the present study are more in keeping with that of Fuller et al. (I979).

The cause of these alterations in the coagulation system is not known. One possibility which does not seem to have been explored systematically is that the degree of metabolic control influences the results. The present study was not designed to answer this point; Part V of this Thesis provides evidence that levels of certain coagulation factors are at least partly dependent on the degree of glycaemic control of the diabetes.

Radiolabelling experiments have shown that fibrinogen consumption is increased in diabetic patients (Ferguson et al., I975; Jones & Peterson,I979). This shortened fibrinogen survival was prolonged when hyperglycaemia was controlled, but fibrinogen taken and labelled during the hyperglycaemic period had a near-normal survival when infused during euglycaemic conditions, suggesting that the fibrinogen molecule is not itself modified during hyperglycaemia. Interestingly, Coller et al.(I978) have described a correlation between the concentration of fibrinogen and the level of Hb A_I. Since the fibrinolytic system appears to be attenuated in diabetes (see Part II, Section 3) the cause of the increased fibrinogen disappearance is not certain, though the overall catabolic rate of plasma proteins appears to be increased in diabetics (Parving et al., I975) and increased vascular permeability might also play a role (Parving, I976) in increasing the rate of clearance. Increased fibrin formation and accelerated fibrin degradation in diabetes is suggested by the finding of increased soluble fibrin complexes (Tsianos & Stathakis, I980). Measurement of the kinetics of other coagulation factors has not yet been applied to the study of thromboembolic disorders, but could yield additional information on the mechanisms of coagulation factor abnormalities in diabetes.

The biological significance of the changes described in this Section are uncertain. Raised fibrinogen levels may be important in increasing whole blood viscosity in diabetic patients (McMillan, I976). Raised levels of individual coagulation factors are generally not believed to indicate a tendency to arterial thrombosis (Davis & McNicol, I978; Hirsh, I98I). However, in a large prospective study, Meade et al. (I980) have shown that factor VIII:C, fibrinogen and particularly factor VII were significantly increased in subjects dying of cardiovascular causes compared to survivors. It is of interest that these same factors were found to be increased in the present study. Prospective trials are required to determine if these alterations are of predictive value in the development of diabetic vascular complications.

SECTION 3 THE FIBRINOLYTIC SYSTEM AND DIABETES MELLITUS

INTRODUCTION

The fibrinolytic system has been the subject of a number of studies in diabetic patients, though results show a remarkable lack of unanimity. Hathorn et al. (I96I), Fearnley et al. (I963) and Almer and Nilsson (I975) have reported low levels of fibrinolytic activity as measured by euglobulin clot lysis or fibrin plate techniques. On the other hand, others (Denborough & Paterson, I962; McKay & Hume, I964; Tanser, I967) found no differences between diabetics and controls whereas Cash and McGill (I969) found increased spontaneous fibrinolytic activity in young insulin-dependent diabetics.

When the fibrinolytic system is stressed, by excercise (Cash & McGill, I969), subcutaneous injection of adrenaline (Tanser, I967) or venous occlusion (Almer & Nilsson, I975), the normal rise in fibrinolytic activity appears to be reduced in diabetic subjects and using a histochemical technique, Almer and Nilsson(I975) have reported that biopsies of arteries and veins from diabetic patients are deficient in vascular activator.

In addition to plasminogen activator production, other abnormalities of components of the fibrinolytic enzyme system have been reported. Concentrations of the antiplasmins α_2-macroglobulin and α_1-antitrypsin have been reported to be raised in diabetic subjects (Ganrot et al., I967; Almer & Nilsson, I975; Jonsson & Wales, I976). Antithrombin III, which is more important for its inhibition of activation of the coagulation system (Part I, Section 3) has been variously reported as low (Banerjee et al., I974), increased (Fuller et al., I979) or normal (Zucker et al., I979) in diabetic patients. The majority of these studies measured only one or two of these components. The aim of the present study was to measure a wide screen of factors invoved in fibrinolysis.

MATERIALS AND METHODS

SUBJECTS

Diabetics and controls were those previously described in the preceeding Sections.

REAGENTS

Casein (Hammerstein quality, Hopkins & Williams, Chadwell Heath, Essex) was prepared as a 4% solution by suspending 4g in 70ml borate-saline buffer, pH 7.4. Two ml IN sodium hydroxide was added and the solution was stirred until the casein was dissolved. The pH was adjusted to 7.4 with IN sodium hydroxide and the volume made up to I00ml with borate-saline buffer. The solution was stored at 4°C for up to one week.

Tyrosine Standard was obtained from BDH Chemicals Ltd.

Streptokinase (Varidase, Lederle Laboratories, New York) was dissolved in 0.I5M saline to give a concentration of 2,000 Christensen u/ml. The solution was stored in small aliquots at - 20°C until use.

Borate-Saline Buffer was prepared by dissolving 4.77g of disodium borate in 250ml distilled water (solution A). I2.40g boric acid and 2.92g sodium chloride were dissolved in distilled water and made up to Il (solution B). Approximately 20ml solution A were added to solution B and the proportions adjusted to give a pH of 7.4.

ASSAYS OF THE FIBRINOLYTIC SYSTEM

Euglobulin Clot Lysis Time (ELT) was based on the method described by Nilsson and Olow (I962). 0.5ml fresh citrated plasma and 9.5ml 0.0I4% acetic acid were added to a glass tube, the contents mixed and stood on ice for IOmin. The resulting precipitate was centrifuged at I000 g for 5 min, the supernatant decanted off and the euglobulin precipitate resuspended by the addition of 0.I5ml Tris buffer (pH 7.4) and emulsification using an orange stick. 0.I5ml of the suspension was placed in a glass tube and 0.I5ml bovine thrombin was added (2 NIHu/ml). The contents were mixed and the tube transferred to a time recorder (Carmanan Instruments Ltd., Glasgow). The time taken for the clot to lyse was taken as the ELT. Results were expressed using a line obtained by plotting the log of the lysis time in min against arbitrary

units of fibrinolytic activity, IOu being equated with a lysis time of 50 min (Sherry et al., I959).

Plasminogen was estimated by a modification (Alkjaersig et al., I959a) of the caseinolytic method of Remmert and Cohen (I949). 0.5ml 0.6N hydrochloric acid was added to 0.5ml oxalated plasma and the mixture was left to stand at room temperature for I5 min. 0.5ml 0.6N sodium hydroxide, Iml borate-saline buffer (pH 7.4), 0.5ml streptokinase (2,000u/ml) and 2ml 4% casein were added and mixed thoroughly. Two ml samples were removed at 2 and 62 min and added to 2ml I0% trichloroacetic acid to precipitate the unhydrolyzed casein. The precipitate was left for 20 min and then centrifuged at I,000 g for 5 min. One ml supernatant was removed and mixed in a glass tube with I.5ml 5% trichloroacetic acid and 5ml 0.5N sodium hydroxide. I.5ml Folin Ciocalteu's reagent was added and the mixture allowed to stand for I5 min. The optical density of the 2 min sample was read against the 62 min sample at a wavelength of 680nm. With each batch of plasmas, Iml of a standard solution containing 400ug/ml tyrosine in place of plasma and hydrochloric acid was also processed. Results were expressed in casein units (Alkjaersig et al., I959b), one unit being the amount of proteolytic enzyme which produces I80ug of tyrosine-like material from the casein in 60 min.

α_I-Antitrypsin, α_2-Macroglobulin, C_I-Esterase Inhibitor and Antithrombin III were assayed by the quantitative single radial immunodiffusion technique (Mancini et al., I965). Commercially prepared immunodiffusion plates and standardized reference sera were obtained from Behringwerke AG. Serum was used for estimations of α_2-macroglobulin and c_I-esterase inhibitor and oxalated plasma for α_I-antitrypsin and antithrombin III assays. The standard serumor plasma was diluted with 0.I5M saline to give three concentrations of known antigen, from which a standard curve was obtained. 5ul of each sample to be tested was pipetted into a well on the immunodiffusion plate, which was left covered at room temperature when the area of precipitation was measured, read off the standard curve, results being expressed as mg/dl.

STATISTICS

The statistical techniques were the same as those used in the preceeding Section.

RESULTS

The results are summarized in Table 7.

ELT

The mean resting ELT was not significantly different between diabetics and controls, and there were no significant differences between the different diabetic sub groups. However, in diabetic subjects, ELT was found to be negatively correlated with obesity index (Table 8).

α_1-antitrypsin

The concentrations of α_1-antitrypsin in diabetics did not differ significantly from controls. Levels were not significantly related to the presence of vascular complications, type of treatment, obesity index, duration of diabetes or age.

α_2-macroglobulin

Levels were significantly higher in the diabetics compared with the controls ($p < 0.05$). There were no significant differences between those with and without vascular complications. Concentrations were significantly higher in insulin dependent compared with non-insulin dependent diabetics ($p < 0.01$) The age of the patient was inversely correlated with the α_2-macroglobulin level (Table 8).

Antithrombin III

Diabetics had significantly lower levels than controls ($p < 0.001$), but this abnormality appeared to be unrelated to the presence of vascular disease. Concentrations of antithrombin III were significantly lower in non-insulin dependent diabetics compared to those on insulin ($p < 0.02$). Levels were also inversely correlated with age (Table 8).

C_1-esterase inhibitor

Levels were significantly higher in diabetics ($p < 0.001$), but this increase appeared to be unrelated to the presence of vascular disease , age, duration of diabetes, treatment or obesity.

Plasminogen

No significant differences between diabetics and controls, or between any of the diabetic sub groups, were observed.

DISCUSSION

This study has shown that the diabetic subjects had significantly increased concentrations of α_2-macroglobulin and c_I-esterase inhibitor and reduced levels of immunologically detectable antithrombin III compared to controls. In contrast, resting ELT and levels of α_I-antitrypsin were not significantly different from controls.

The finding of increased α_2-macroglobulin concentrations in diabetics is in agreement with others (Ganrot, 1967; Almer & Nilsson, 1975; Jonsson & Wales, 1976), but a new observation is that levels were higher in insulin dependent diabetics compared to non-insulin dependent patients. Though α_2-macroglobulin has been shown to increase with age in normal subjects (Hamilton et al., 1974b), in the present study diabetics showed an inverse relationship, presumably because of the lower age of the insulin dependent patients. The relationship of α_2-macroglobulin concentrations with age is shown in Fig. II.

Raised levels of c_I esterase inhibitor have not previously been reported in diabetes. This elevation was not related to age of the patients, but appeared to be related to the presence of vascular complications.

The lower levels of antithrombin III may be explained by the greater age of the diabetics compared to controls since there was a significant negative correlation with age in the diabetic group. However, it should be noted that like α_2-macroglobulin, abnormal levels were found in insulin-dependent diabetics compared to those on oral therapy.

ELT was not significantly different in diabetics compared to controls. From the results, it can be seen that the standard deviations for this test were wide. It is possible that some of the abnormal results of resting ELT reported by others may be caused by a preponderance of obese subjects in the diabetic group, since the present study confirms earlier reports that obese subjects have lower fibrinolytic activity (Ogston & McAndrew, 1964). It may also be that release of fibrinolytic activators after a stimulus such as venous occlusion may be a more valid test of overall firinolytic potential.

As with the coagulation system, the reasons for the changes found in this and other studies is uncertain. Plasma proteins such as fibrinogen, factor VIII, and α_1-antitrypsin rise, and antithrombin III levels fall following a wide variety of conditions involving tissue injury and are commonly referred to as acute phase reactants. Unfortunately, use of this term obscures the fact that it is not known by what mechanisms such changes take place. In diabetes, such changes could be merely secondary to an injured vascular tree (caused by atheroma or microangiopathy) Alternatively, there may be a more direct metabolic explanation, since it has been shown that in isolated rat liver experiments, infusions of insulin, cortisol and growth hormone in physiolological quantities increased the synthesis of fibrinogen and α_1 and α_2-glycoproteins, suggesting that hormonal factors may be of importance (John & Miller, 1969).

Whether such changes have any influence on the development of vascular complications is also unknown. C_1-esterase inhibitor is a relatively minor antiplasmin (Part I, Section 3) and patients with inherited antithrombin III deficiency have levels less than 60% of normal before thrombotic problems are encountered (Marciniak et al., 1974) so that it may be more logical to regard these abnormalities as markers of tissue damage rather than factors of pathogenetic significance. On the other hand, α_2-macroglobulin is responsible for a considerable proportion of the increased whole blood viscosity found in diabetics (Skovberg et al., 1966) and it has been suggested that one effect of the increased concentrations of α_2-macroglobulin in diabetics is to inhibit the action of leukocyte proteases which normally degrade the basement membrane, thus contributing to the thickening characteristic of microangiopathy (Brownlee, 1976).

PART III PLATELET MEMBRANE ABNORMALITIES IN DIABETES MELLITUS

SECTION I PLATELET SURVIVAL IN DIABETES MELLITUS

PLATELET SURVIVAL

In vitro tests of platelet function may be criticized since they are performed under unphysiological conditions and their relevance to platelet behaviour in vivo is uncertain. An alternative approach has been to study the disappearance of radiolabelled platelets from the circulation. Two radioisotopic approaches have been employed in the study of platelet survival. In one, a cohort of platelets is labelled; in the other, a mixed population of circulating platelets is labelled (a 'random' or 'population' label).

Though cohort labelling should theoretically produce more accurate and easily analyzed data, no satisfactory cohort label exists for platelets. Attempts with ^{32}P, ^{32}P-diisopropyl fluorophosphate ($DF^{32}P$), ^{35}S or ^{75}Selenomethionine have not proved satisfactory, since the period which they remain available for labelling megakaryocytes is long compared to platelet lifespan (for reviews see Aster, I97I; Gardner, I972; Harker, I978). Thus labelled platelets continue to appear during the time platelets are being removed from circulation, making results difficult to interprete.

Random labels, such as ^{32}P-orthophosphate , DFP labelled with ^{32}P, ^{3}H and ^{14}C and ^{14}C-serotonin have been extensively tested, but problems with elution or reutilization of the label, or toxicity to the subjects has led to most investigators abandoning these techniques in human studies. In I977, an international committee concluded that ^{51}Cr-sodium chromate was the only isotope suitable for platelet survival studies (Panel on Diagnostic Applications of Radioisotopes in Hematology, I977). Since this date, ^{111}In, complexed with hydroxyquinoline has been used as a platelet marker, with the advantages over ^{51}Cr that much smaller quantities of blood need to be removed for platelet labelling - as little as 26ml (Hawker et al., I978), labelling efficiency appears to be superior to ^{51}Cr (Scheffel et al., I977) and finally the high energy permits the in vivo imaging of labelled platelets using a gamma camera (Scheffel et al., I977). However, the relative high cost, the short half-life of the isotope (2.8 days) and the problems associated with the high energy radiation has limited the use of ^{111}In to specialized centres, and ^{51}Cr is at present used by the majority of laboratories measuring platelet survival. The method first described by Aas and Gardner (I958) was subsequently modified by Aster and Jandl (I964), resulting

in improved recovery and viability of the labelled platelets.

Though widely used, the technique has important limitations. Repeated estimations of ^{51}Cr-labelled platelet survival are not desirable because of radiation hazards and are contraindicated during pregnancy. A minimum of 100ml of blood must be removed to obtain sufficient platelets for labelling (Abrahamsen, 1968a). The proceedure of labelling requires considerable attention to detail to avoid platelet damage and is time consuming (in the author's experience the proceedure takes at least 3h). Veins may be compromized owing to the large cannula required to remove the blood (of particular importance in diabetic patients where access to veins is frequently difficult). Finally, facilities for handling radioisotopes are required.

ASPIRIN LABELLING TECHNIQUE

First described by Stuart et al. (1975), this method appeared to offer an attractive alternative to established techniques. The procedure makes use of the fact that acetyl salicylic acid irreversibly inhibits the capacity of the platelet to convert arachidonic acid to the endoperoxides PGG_2 and PGH_2 (see Part I, Section 2), by acetylating platelet cyclo-oxygenase (Roth et al., 1975). This inhibition persists throughout the life of the platelet. As a by-product of thromboxanes produced from the cyclic endoperoxides, malondialdehyde (MDA) is formed (Smith et al., 1976), which can be estimated colorimetrically by reaction with thiobarbituric acid (Sinnhuber & Yu, 1958). Reappearance of MDA should therefore parallel the entry of new platelets into the circulation unlabelled with aspirin.

The present study was designed to evaluate this new method and to compare platelet survival in diabetic and control subjects.

MATERIALS

N-ethylmaleimide (Sigma Chemical Co.) was dissolved in phosphate-saline buffer, pH 7.4 to give a 20mM solution. Aliquots were placed in polythene tubes and stored at - 20°C until use.

Phosphate-Saline Buffer was prepared by mixing 18ml solution A (23.4g sodium dihydrogen phosphate/1 distilled water) with 82 ml solution B (21.3g disodium hydrogen phosphate/1 distilled water). An equal volume of 0.15M saline was added to produce a buffer of 0.15M and a pH of 7.4.

Sodium Thiobarbiturate was produced by dissolving 500mg 2-thiobarbituric acid (Sigma Chemical Co.) in 10ml 2.5N sodium hydroxide with gentle warming. The pH was adjusted to 7.4 with 1N hydrochloric acid and the volume was made up to 50ml with distilled water. The solution was stored out of the light and was made up fresh for each estimation of platelet survival.

Magnetic Stirrers were made from cylindrical magnets 3mm long x 2mm diameter (Sheffield Magnet Co., Sheffield). The magnet was placed inside a 3mm length of polythene tubing gauge No. BP 270 (Portex Ltd., Kent) and the ends were sealed with silicone rubber solution (Dow Corning Corp.).

1,1,3,3-tetraethoxypropane (TEP) was obtained from BDH Ltd.

STANDARD CURVE FOR MALONDIALDEHYDE

METHOD

The principle of the procedure employs the fact that acid hydrolysis of I mole of I,I,3,3-tetraethoxypropane (TEP) yields one mole of MDA and four moles of ethanol (Sinnhuber & Yu, I958).

The method used was a modification of that described by Kwon & Watts (I963). TEP was not subjected to overnight hydrolysis since MDA polymers may form, with loss of reactivity with thiobarbituric acid (Gutteridge, I975). 220.49mg TEP were added to a Il stoppered glass flask. One ml IN hydrochloric acid was added and the volume was made up to exactly Il with distilled water, giving a concentration of TEP of I x IO^{-3}M. This stock solution was stored at 4°C until use.

Dilutions of the stock solution were made with distilled water, to give final concentrations ranging from 0.428 to 0.005 x IO^{-5}M. Duplicate Iml samples of each concentration were placed in glass centrifuge tubes and Iml of distilled water treated in the same way acted as blank. 0.2ml 20% aqueous solution of trichloroacetic acid and 0.2ml of the sodium thiobarbiturate solution were added. The tubes were agitated , boiled in a water bath (marbles were used as condensors) for I5 min, cooled and centrifuged at I,700 g for I0 min. The optical density of the solutions was read at 532nm against distilled water, using a Pye Unicam SP I800 spectrophotometer.

RESULTS AND COMMENTS

The results of a typical experiment are shown in Table 9. When the concentration of MDA was plotted against optical density, a linear curve was obtained throughout the range of concentrations used, with the line passing through zero (Fig. I2).

The molar absorptivity ε (defined as the optical density produced by one mole in a one cm light path) was calculated from the curve to be 1.55×10^5, which corresponds exactly to that obtained by Placer et al (1966) for MDA. The amount of MDA in a sample may therefore be calculated by the formula :

$$\text{MDA (nmol)} = \frac{V \times OD_{532}}{0.155}$$

(Placer et al., 1966)

where V is the final volume of the test solution, OD_{532} is the optical density at 532nm .

However, since the standard curve was linear within the limits of optical density used in the platelet survival experiments, optical density was used to compare the results of MDA assays, results being expressed as $OD/10^9$ platelets.

EFFECT OF STORAGE OF REAGENTS IN MALONDIALDEHYDE ASSAY

INTRODUCTION

The assay system as originally described by Stuart et al. (1975) was found to be unsatisfactory. Using the mixture of perchloric acid and thiobarbituric acid, the final pink solution was frequently turbid in spite of prolonged centrifugation, making optical density readings unreliable. The same problem was also described by Okuma et al. (1971) who clarified the solution with addition of alkali and Biosolv, a commercial solvent. This has the disadvantages of diluting the chromagen (resulting in loss of sensitivity) and also increases its lability (Okuma et al., 1971). Placer et al. (1966) overcame the problem by adding a pyridine/n-butanol mixture but this is volatile, emitting toxic fumes. A further consideration is that Gutteridge et al. (1974) have shown that when fatty acids are boiled with thiobarbituric acid and percloric acid (as in the Stuart assay) MDA may be formed in the absence of an oxidating agent such as n-ethyl maleimide.

To avoid these problems, a modification of the method of Flower et al. (1973) was used. In this technique, trichloroacetic acid is substituted for the perchloric acid to precipitate the protein. To avoid auto-oxidation of fatty acids, the platelet proteins were first precipitated with the trichloroacetic acid. The solution was centrifuged and the supernatant was boiled with the sodium thiobarbiturate. No problems with turbidity were encountered with this method.

The following experiment was designed to determine whether the modified reagents used in the MDA assay, and the standard solution of MDA derived from TEP were stable on storage.

METHODS

One per cent sodium thiobarbiturate and 20% trichloroacetic acid were kept in stoppered glass bottles at room temperature. A solution of MDA (0.3×10^{-5}M) was made from TEP as previously described and was stored throughout the experiment at 4°C. The MDA assay was performed in duplicate as described in the previous experiment. At daily intervals, the assay was repeated, using the same solution of MDA and the same reagents.

RESULTS AND COMMENTS

The results over a two week period are shown in Table 10. The resulting chromagen did not decline over the two week period, suggesting that the reagents are sufficiently stable over this time.

Thus, one batch of reagents is adequate to measure platelet survival. During this time, the sodium barbiturate produced a white-brown precipitate. However, this did not effect the efficiency of the assay. The coefficient of variance for this experiment was 1.3%, suggesting that the method is highly reproducible.

DAY-TO-DAY VARIABILITY IN PLATELET MALONDIALDEHYDE PRODUCTION

INTRODUCTION

The accurate estimation of platelet lifespan by the MDA technique depends on a reliable baseline before the administration of aspirin. The following experiment was designed to assess day-to-day variability in platelet MDA production by normal subjects.

SUBJECTS

Four subjects (2 men, 2 women) volunteered to take part in the study. None had taken drugs within the preceeding two weeks and were in apparent good health. The women were not menstruating at the time of the study.

METHODS

Nine ml of venous blood was removed from each subject at intervals of several days and placed in a plastic tube containing Iml 0.077M EDTA (ethylenediamine tetraacetic acid, pH 7.4). The blood was gently mixed and centrifuged at 250 g for IO min to obtain PRP, which was removed using a plastic pipette. The platelet count of the PRP was performed as previously described. Duplicate Iml samples were placed in polypropylene conical centrifuge tubes and centrifuged at IOOO g for I2.5 min to obtain platelet buttons. The supernatant was removed by inversion of the tubes which were allowed to stand for 2 min to drain off the remaining PRP. The sides of the tubes were carefully dried with filter paper and Iml phosphate buffer, pH 7.4, added to each tube. The tubes were placed in a waterbath at 37°C which was placed above a magnetic stirrer. The polythene-coated magnets were used to resuspend the platelets for 4 min then 0.Iml 20mM n-ethyl maleimide was added and the tubes stirred for a further minute. The magnets were then removed and the tubes maintained at 37°C for exactly 30 min, when the reaction was stopped by the addition of 0.2ml 20% trichloroacetic acid. The tubes were centrifuged for I5 min at I,000 g and Iml of the supernatant was placed in a glass centrifuge tube. 0.2ml of I% sodium thiobarbiturate was added and the tubes were placed in a boiling waterbath for I5 min, cooled and centrifuged at I,000 g for a further IO min. The optical density of the resulting pink solution was read at 532nm, using a Pye Unicam SP I800 and Icm light path silica cells against distilled water. For each estimation, a reagent blank was

also read. Results were expressed as optical density/10^9 platelets.

RESULTS AND COMMENTS

The results of five sets of estimations spread over one week are shown in Table II. The coefficients of variation for the four subjects were 2.22%, 2.77%, 5.20% and 5.00% respectively. It can be seen that though inter-individual differences between subjects were quite marked, MDA production remained constant for each individual. Variability was approximately twice as high in the two female subjects as in the males. This prompted a study on the influence of the menstrual study on platelet MDA production (Tindall et al., 1981a) which found that production drops by approximately one third following the onset of menstruation, rising back to normal after three to four days. The reason for these changes is not known, but does impose a serious limitation on this method of measuring platelet lifespan.

PLATELET SURVIVAL IN DIABETIC PATIENTS USING AN ASPIRIN LABELLING TECHNIQUE

INTRODUCTION

Platelet survival has been reported to be shortened in a variety of thromboembolic conditions including acute (Abrahamsen, I968b) and recurrent venous thrombosis (Harker & Slichter, I974), rheumatic valve disease (Steele et al., I974), acute myocardial infarction (Abrahamsen, I968b) and following replacement of diseased heart valves with the older types of prosthetic valves (Harker & Slichter, I972, I974; Weily et al., I974). In this latter study, shortened platelet survival was related to the frequency of embolic events. In stable ischaemic heart disease, results have been less consistent, with normal (Abrahamsen, I968b; Kutti & Weinfeld, I97I) and shortened (Murphy & Mustard, I962) platelet survival having been described. Two recent studies have shown that platelet survival is shortened in the presence of angiographically proven coronary artery disease (Steele et al., I973; Richie & Harker, I977).

Since measurement of platelet survival would appear to be a useful method to detect an increased tendency to arterial thromboembolism, it is perhaps surprising that few investigators have used this technique in diabetic patients. Abrahamsen (I968b) studied 28 patients with maturity onset and juvenile onset diabetes, using ^{5I}Cr. Maturity onset diabetics did not differ from non diabetic controls; juvenile onset patients had slightly reduced survival times and those with micro- or macrovascular complications had significantly shorter platelet survival times than controls. Ferguson et al. (I975), using ^{75}Se, studied eight insulin dependent diabetics without vascular complications, and also found platelet survival to be shortened compared to controls. Finally, Dassin et al. (I978) found increased platelet consumption in one third of 3I insulin dependent diabetics using ^{5I}Cr, but this shortening could not be related to the degree of vascular complications.

The present study measured platelet survival in twelve diabetics and twelve controls, using the aspirin technique.

MATERIALS AND METHODS

Subjects

Twelve non-diabetic controls (7 men, 5 women) and I2 diabetics (7 men, 5 women) were studied. The controls were healthy laboratory and medical staff with a mean age of 30.8 years (range 23-49 years). The diabetics were clinical diabetics using the criteria of Fitzgerald and Keen (I964). The mean age was 39.4 years (range I6-65). Clinical details of the diabetic patients are shown on Table I2. None of the subjects was taking any medications known to influence platelet behaviour, and in particular, aspirin-containing drugs were avoided for at least two weeks prior to the study. None of the women were menstruating during estimation of platelet survival. All blood samples were removed under non-fasting conditions (MDA production does not alter with a rise in plasma glucose (Tindall et al., I98Ib).

Methods

MDA production of n-ethyl maleimide stimulated platelets was performed as described in the preceeding Chapter. The subject was then given 600mg soluble aspirin and MDA production was measured at daily intervals until values exceeded 80% of pre-aspirin levels. Results were expressed as a percentage of the pre-aspirin level and plotted against time in days. For each subject, a straight line was computed using linear regression analysis and the platelet survival time was expressed as the time in days an extrapolation of the line intersected I00% of the pre-aspirin level (Fig I3).

Statistics

Platelet survival times were compared using Wilcoxon's rank sign test for unpaired data. Correlations were calculated using linear regression analysis.

RESULTS

As can be seen from Fig. I4, recovery of MDA production after the first day was linear up to 80% of pre-aspirin levels in both diabetics and controls. As shown in Fig. I5, platelet survival of the diabetic patients was significantly shorter than that of the controls ($p < 0.01$), the mean values being 6.92 days and 9.05 days respectively. Shortened platelet survival did not seem to be related to the presence of vascular complications as mean platelet survival time of the six diabetics without vascular complications were not significantly different from those with vascular disease (Table I3). Shortened platelet survival was not significantly correlated with age of the diabetic (r -0.067, $p > 0.05$) or duration of diabetes (r 0.263, $p > 0.05$).

DISCUSSION

Using a non-isotopic method for measurement of platelet survival, survival times were significantly shorter than those of controls. This finding seemed not to be related to the presence of vascular complications or to the duration of diabetes, though the numbers of patients studied was too small for detailed statistical analysis. The mean age of the diabetics was slightly greater than the controls. Platelet survival has been reported to decrease with age (Abrahamsen, I968b), but this would seem to be an unlikely explanation of the present findings since in this study, no significant correlation with age was found and one of the shortest platelet survivals in the series was in a 20 year old diabetic. Assessment of metabolic control was not made in this study; it remains speculative that shortened platelet survival is related to poor metabolic control.

Advantages of the aspirin-labelling technique include its simplicity, low cost and, since it does not expose the subject under study to radioactivity, may be particularly useful in children and during pregnancy, and for studies which demand repeated measurements of platelet survival. The results obtained correlate reasonably closely with the standard ^{51}Cr method (de Haas et al., I979; Roncucci et al., I979; Tindall et al., I98Ic) in normal and thromboembolic conditions.

Since the aspirin-labelling method actually measures the rate of platelet production rather than platelet removal, this technique

reflects platelet survival when the rate of the two processes is the same, that is, when the platelet count remains constant. Some authors have preferred to refer to the aspirin-labelling technique as "platelet regeneration time" (Roncucci et al., I979) or "platelet production time" (de Haas et al., I979). In this paper, the term platelet survival has been retained to simplify the terminology.

Unlike the radioisotopic methods, the aspirin-labelling technique can not be used when a subject is taking drugs which interfere with MDA production (e.g. non-steroidal antiinflammatory drugs), though recently Tindall et al. (I98Ic) have shown that valid platelet survival results can be obtained if recovery of MDA production is measured immediately after discontinuation of the drug. Under these conditions, the results obtained appear to refer to the survival of platelets during the period of drug administration.

A more practical problem of the technique is that the sensitivity of the colorimetric assay is relatively low. As can be seen from Fig. I2, the limits of detection are reached at approximately IOpmol/ml and the useful range is between 50 - 400pmol/ml. Consequently, a washed platelet system must be used since in the presence of plasma proteins MDA production is undetectable (personal observation; Catalano et al., I98I). Modifications of the method have been introduced by a number of investigators. The substitution of arachidonic acid for n-ethyl maleimide has been reported to increase the sensitivity of the assay IO-fold (de Haas et al., I979) but platelet washing is still required. The fluorometric assay of MDA described by McMillan et al. (I977) has permitted the measurement of MDA in PRP after thrombin-stimulation, thus avoiding possible artefacts during a washing procedure (Tindall et al., I98Ib).

The cause of shortened platelet survival in diabetes is not known. Previous studies have been in agreement with the present results which found no clear relation to the presence of clinically detectable vascular disease. In an extension of the present study, Tindall et al. (I98Ib) found that a group of 40 insulin-dependent diabetics (20 with severe retinopathy and 20 with minor background changes only) had highly significantly shorter platelet survival times than 40 controls, but there was no statistical difference between the two diabetic sub-groups. These results, as with the present study, may be interpreted as either clinically undetected vascular damage or potentially reversible metabolic factors are responsible for these changes. In this regard, it has recently been shown that shortened fibrinogen survival in diabetics can be prolonged by tight control of hyperglycaemia (Jones & Peterson, I979).

As with the causes of the shortened platelet survival, the mechanism of removal of platelets prematurely from the circulation in diabetes and other thromboembolic conditions is incompletely understood. Reimers et al. (1973) have shown that thrombin-degranulated rabbit platelets have a normal lifespan. This suggests that platelets can undergo reversible aggregation in vivo and continue to circulate normally. Changes of the platelet membrane seem more important in determining lifespan. When sialic acid was removed from the surface glycoproteins of rabbit platelets, platelet survival was markedly reduced (Greenberg et al., 1975). Experiments by George et al. (1976, 1978) show that when surface glycoproteins are marked with ^{125}I-diazotized diiodosulfanilic acid, radioactivity is lost more rapidly than ^{51}Cr. This apparent membrane loss may occur during reversible adhesion to the vessel wall and thus modify the platelet so that it is removed from circulation by the reticuloendothelial system. If this hypothesis is correct, it would appear logical to search for membrane abnormalities in the platelets of diabetic subjects, which was the aim of the next experiments reported in this Thesis.

SECTION 2 FIBRINOGEN BINDING AND ADP-INDUCED AGGREGATION IN PLATELETS FROM DIABETIC SUBJECTS

FIBRINOGEN BINDING AND ADP-INDUCED AGGREGATION IN PLATELETS FROM DIABETIC SUBJECTS

INTRODUCTION

Evidence that in vitro platelet aggregation is increased in certain groups of diabetic subjects has been presented in Part II, Section I of this Thesis. However, what causes this hyperaggregability is not known. A plasma factor, found in plasma from diabetic subjects, which enhanced the second phase of ADP-induced aggregation when added to control PRP has been described (Kwaan et al., I972a; Leone et al., I974; Colwell et al., I977). On the other hand, these observations have not been confirmed by others, who concluded that the abnormality was intrinsic to the platelet itself (Bensoussan et al., I975; Coller et al., I978

It has been stated that only the second phase of ADP-induced platelet aggregation is enhanced in diabetes (Colwell et al., I978); it is certainly this aspect of platelet aggregation that has received most attention, and it has been related to an increased production of proaggregatory arachidonic acid derivatives such as thromboxane A_2 (Ziboh et al., I979; Halushka et al., I98I). Recently, an increase in ADP-induced platelet shape change has been described in diabetics, particularly those with retinopathy (Porta et al., I980) which implies that an earlier stage in platelet activation may also be affected.

Fibrinogen is an essential co-factor for ADP-induced aggegation of human platelets (Born & Cross, I964; Niewiarowski et al., I977). Mustard et al. (I978) demonstrated that ^{I25}I-fibrinogen became associated with human platelets during ADP-induced shape change and aggregation. Subsequent studies demonstrated the existence of specific and saturable receptors for fibrinogen which became exposed on the platelet surface in the presence of ADP or adrenaline, and that the binding required divalent cations such as calcium or magnesium (Marguerie et al., I979, I980; Peerschke et al., I980; Bennett & Vilaire, I979). Peerschke et al. (I980) have proposed that a relationship exists between the quantity of fibrinogen bound and platelet aggregability. This study investigated the possibility that the hyperaggregability of platelets from patients with severe retinopathy is related to increased fibrinogen binding.

MATERIALS AND METHODS

SUBJECTS

Sixteen diabetics (I2 men, 4 women) were selected from those patients attending the Diabetic Clinic at Hôpital St Louis. Clinical details of the patients are shown in Table I4. The mean age was 40.9 years (range 2I - 42 years). The extent of retinopathy was assessed by retinal fundoscopy and fluorescein angiography. The diabetics were separated into two groups on the basis of retinal appearances : eight had no or minimal retinal changes (less than five microaneurysms in each eye) and were termed "background retinopathy". Eight were chosen because of severe retinopathy which required photocoagulation treatment (one maculopathy and seven with proliferative retinopathy). Diagnosis of diabetes was based on the criteria of Keen et al(I979)

Nine non-diabetic controls (7 men, 2 women) were selected from medical and laboratory staff. Mean age was 27.2 years (range 24 - 42 years). All were in good health at the time of study and had no family history of diabetes.

Neither diabetics nor controls were receiving any drugs known to affect platelet function for two weeks prior to the tests. None of the female subjects were taking oral contraceptives.

REAGENTS

Acid Citrate Dextrose was prepared by dissolving 0.8g citric acid, 2.2g trisodium citrate and 2.45g glucose in distilled water and making the volume up to I00ml .

Platelet Washing Buffer was made by dissolving 7.56g citric acid, 0.9g glucose, 0.373g potassium chloride, 0.294g calcium chloride ($2H_2O$), 0.203g magnesium chloride and 6g sodium chloride in 700ml distilled water. The pH was adjusted to 6.5 with 2N sodium hydroxide and the volume made up to Il with distilled water to give a buffer containing 36mM citric acid, 5mM glucose, 5mM potassium chloride, 2mM calcium chloride, ImM magnesium chloride and I03mM sodium chloride.

Reaction Buffer was made by dissolving 8g sodium chloride, 0.2g potassium chloride, Ig sodium bicarbonate, 0.05g sodium dihydrogen phosphate, 0.43g calcium chloride ($2H_2O$), 0.203g magnesium chloride and Ig glucose in 700ml distilled water. The pH was adjusted to 7.35

with IN hydrochloric acid and made up to Il with distilled water, giving a buffer containing I37mM sodium chloride, 2.7mM potassium chloride, IOmM sodium bicarbonate, 0.3mM sodium dihydrogen, 2mM calcium chloride, ImM magnesium chloride and 5.5mM glucose.

Bovine Serum Albumin, Cohn fraction V, was obtained from Sigma Chemical Co. The albumin was dissolved in reaction buffer to give a stock solution of 3.5mg/ml which was stored in small aliquots at - 20°C until use.

Apyrase was obtained from Sigma Chemical Co. It was dissolved in the washing buffer to give a solution of 5mg/ml. Each lot was tested before use, since some lots possessed platelet aggregatory properties due to impurities. The stock solution of apyrase was divided into small aliquots and frozen at - 20°C.

Prostaglandin E_I (PGE_I) was a gift from Dr J. Pike, the Upjohn Co., Michigan. The PGE_I was originally stored at - 20°C in 70% ethanol 30% water at a concentration of Img/ml. This solution was diluted to a concentration of 7ug/ml in the washing buffer and frozen in small aliquots at - 20°C.

Heparin (Choay, Paris) was kept at 4°C, at a concentration of 5,000U/ml.

Adenosine 5' Diphosphate Grade I Sodium Salt (Sigma Chemical Co.) was dissolved in Tris buffer (0.I5M, pH 7.35) and stored at a concentration of I x IO^{-3}M in small aliquots at - 20°C until required.

ADP stock was changed every three months and the washing and reaction buffers were freshly prepared at weekly intervals.

METHODS

Platelet Separation

After informed consent, blood was removed from all subjects within 2h after breakfast. Diabetics received their normal antidiabetic therapy on the morning of the test. For platelet aggregation studies, PRP was prepared from 20ml citrated blood (0.I28M) by centrifugation at I20 g for IO min. After aspiration of PRP, PPP was produced by centrifuging the remainder at I,200 g for I5 min. The platelet count of the PRP was determined by the method of Brecher and Cronkite (I950) as previously described (Part II, Section I), and the PRP was diluted with PPP to give a platelet count between 250 - 300 x IO^9/l.

Washed platelets for fibrinogen binding studies were produced by taking the blood into acid citrate dextrose in a ratio of 4.5 volumes blood to one volume anticoagulant. Blood was taken into two polythene conical 50ml tubes containing the anticoagulant, 250ul of apyrase stock (final concentration, 25ug/ml) and 50ul of PGE_I stock (final concentration , 20nM). The contents of the tube were mixed carefully and the blood poured into 5ml plastic tubes. PRP was produced by centrifugation at 120g for 10min, pooled in conical plastic tubes (capacity 12ml) and the PRP was centrifuged at 1,200 g for 10 min. The resulting platelet pellet was resuspended by decanting off the PPP and adding 1ml volumes of the washing buffer and agitating gently with an automatic pipette with a polythene tip. Care was taken to leave any erythrocytes at the bottom of the tube. The resuspended platelets were transferred to a fresh conical tube, the volume made up to 10ml with fresh washing buffer and the tube was recentrifuged at 1,200 g for a further 10 min. The first washing buffer contained 5U/ml heparin which was omitted for the second wash. Both washing buffers contained bovine serum albumin in a concentration of 3.5mg/ml. The platelet button was finally resuspended in the reaction buffer containing 3.5mg bovine serum albumin , and the platelet count was adjusted to 1.5×10^6/ul. All manipulations were performed at room temperature.

Platelet Aggregation

Aggregation was performed using a dual-channel aggregometer (Payton Associates, Scarborough, Ontario) adjusted to give a stirring speed of 900rpm. Samples of 0.5ml were used, and the sensitivity of the recorder was adjusted so that PRP and PPP corresponded to 10% and 90% light transmittence respectively. Chart speed was set at 10cm/min. Aggregation was started one h following venesection. 0.5ml samples of PRP were warmed at 37°C for exactly one min in the heating block without stirring. After 45s incubation the sample was stirred by adding a stir bar and at 60s the ADP was added, using a microsyringe. For each experiment, ADP stock was diluted to a concentration of 1.25×10^{-5}M with 0.15M saline. This was added to the PRP in small volumes to give a range of ADP concentrations from 0.5×10^{-6} to 3×10^{-6}M (final concentrations). For example, 10ul added to 500ul PRP was equivalent to a concentration of 2.5×10^{-6}M, 12ul to a concentration of 3.0uM.

Platelet aggregation was followed for 30s, and for each subject, five to seven concentrations of ADP were tested.

Analysis of the results was performed using the method of Zuzel et al. (I979a). For each aggregation curve, a tangent was drawn through the steepest part of the curve (Fig. I6). The reciprocal of the initial rate of aggregation obtained from the tangent was plotted against the reciprocal of the ADP concentration to give a linear dose-response Lineweaver-Burk plot as shown in Fig. I7 (Skoza et al., I967). The intercept on the horizontal axis was used to obtain the reciprocal of the K_m. This constant may be defined as the concentration of ADP required to produce half-maximal aggregation (V_{max}), where V_{max} is the theoretical maximal aggregation rate produced by an infinite concentration of ADP. All aggregation studies were performed within 4h of blood collection.

Fibrinogen Binding

Fibrinogen binding was performed using the method of Lee et al. (I98I). ^{I25}I-radiolabelled fibrinogen was purchased from the Centre Nationale de Transfusion Sanguine (Orsay, France). This fibrinogen had been purified using the method of Jaques (I943),and consisted of greater than 96% clottable protein following the addition of I NIHu of purified thrombin and incubation at 37°C for one hr (Lee et al., I98I). The purified fibrinogen was iodinated with carrier-free (^{I25}) Na I (New England Nuclear Co.) by the chloramine T method (Hunter & Greenwood, I962). The ^{I25}I-fibrinogen was greater than 96% precipitable in 20% trichloracetic acid and approximately 94% clottable with thrombin. Preparations varied between I00 and 300uCi/mg protein. The labelled fibrinogen was diluted 50-fold in reaction buffer containing 3.5mg/ml of bovine serum albumin and frozen at -20°C. Immediately before use, the thawed fibrinogen was centrifuged at I2,000 g for I0 min as a precaution to eliminate aggregates. No contamination of the preparation by factor VIII-related antigen could be detected by immunoelectrophoresis using a monospecific antibody to factor VII-related antigen (Lee et al., I98I). At a concentration of 4mg/ml, the fibrinogen did not support ristocetin-induced platelet aggregation using formalin-fixed platelets and when applied to 7-I2% exponential acrylamide slab gels under reduced conditions, no radioactive bands in the position of reduced factor VIII and cold insoluble globulin were observed (Lee et al., I98I).

Fibrinogen binding to platelets was carried out at room temperature, using freshly-washed platelet suspensions. The incubation mixture contained 360ul of a suspension of platelets in reaction buffer at a concentration of I.5 x IO^6/ul, 450ul of reaction buffer containing 3.5 mg/ml bovine serum albumin, 45ul of ^{I25}I-fibrinogen (300ug/ml) which was immediately followed by 45ul of ADP. The total volume for each point in the assay was therefore 900ul and contained 30mg/ml ^{I25}I-fibrinogen, IOuM ADP and 6 x IO^5 platelets. Upon the addition of ADP (zero time), the solution was gently mixed by swirling and left to stand. Parallel incubations without ADP served as controls for the determination of non-specific binding. At 5, I5, and 30min after the addition of ADP, triplicate samples of 250ul were taken from each reaction mixture and carefully layered onto Iml cold 20% w/w sucrose in reaction buffer containing bovine serum albumin (3.5mg/ml). The tubes were immediately centrifuged at I2,000 g for 2 min in an Eppendorf centrifuge to separate the platelets from the free ^{I25}I-fibrinogen. The supernatant was carefully removed without disturbing the platelet pellet. The radioactivity in each pellet was counted in a CG 4000 Gamma counter (Intertechnique Roche Bioelectronique, Velizy, France). Total radioactivity was calculated from duplicate samples taken from each incubation mixture. The radioactivity that was present in the pellet was expressed as the percentage of the total counts in the 250ul sample of the incubation mixture. Non-specific binding was obtained from parallel samples in which no ADP was added, and subtracted from the total to give specific binding.

Haemoglobin A_I was measured using the micro-column kit produced by Biorad Laboratories (Richmond, California) by the Department of Clinical Chemistry at Hôpital St Louis. The upper limit in non-diabetic subjects in this laboratory is less than 7.5%.

Statistics

Results were compared using Wilcoxon's rank sign for unpaired data. Correlation coefficients were calculated using linear regression analysis.

RESULTS

ADP-induced platelet aggregation

For ease of presentation, the results of platelet aggregation are shown as I/K_m. Thus, the higher the I/K_m, the more sensitive are the platelets to the aggregating effect of ADP. Aggregation was not performed on one control owing to clotting of the blood sample, and in one control and one diabetic (with background retinopathy) non-linear dose response curves were obtained. These results were therefore excluded from the analysis. The I/K_m of the I5 diabetics was significantly increased compared to the controls ($p < 0.05$). The diabetics with severe retinopathy had significantly ($p < 0.05$) higher I/K_m than the controls though differences between controls and diabetics with background retinopathy, and between those diabetics with and without severe retinopathy approached, but did not reach statistical significance (Fig. I8).

I/K_m did not correlate significantly with age (r 0.26), or to duration of diabetes (r 0.40), but there was a highly significant correlation with the concentration of HbA_I as can be seen in Fig. I9 (r 0.79, $p < 0.00I$).

Fibrinogen binding

The mean percentage platelet-bound fibrinogen was higher in diabetics compared with controls at 5, I5 and 30 min after the addition of IOuM ADP (Fig. 20). When the controls were compared with the eight diabetics with severe retinopathy, this difference reached statistical difference at I5 min ($p < 0.05$). Increased fibrinogen binding did not correlate significantly with age of the diabetics (r 0.I0), duration of the diabetes (r 0.0I) or to the concentration of HbA_I (r - 0.25). In addition, fibrinogen binding did not significantly correlate with ADP-induced aggregation, as expressed by I/K_m (r 0.I5). All correlations were calculated with specific fibrinogen binding at I5 min.

DISCUSSION

This study reports the preliminary finding that ^{I25}I-fibrinogen binding to washed platelets is enhanced in diabetes. In common with many other abnormalities of platelet function described in this condition, there was an appreciable overlap in the percentage

of fibrinogen bound between diabetics and non-diabetics, and the increase was most evident in those diabetics with severe retinopathy. The experimental system employed does not permit the distinction between increased fibrinogen binding due to an increased affinity of the platelet receptor for fibrinogen from an increased number of these binding sites. To elucidate this problem, Scatchard analysis of the binding data would be required (Marguerie et al., I979, I980; Bennett & Vilaire, I979). Such studies require considerable quantities of platelets, which are generally obtained from pooled blood bank donations. The present study required I00ml from each subject. Since diabetics have been reported to have increased numbers of megathrombocytes in the peripheral blood (Garg et al., I972, Colwell et al., I977), a shift in the frequency distribution of platelet volume towards larger platelets might result in increased numbers of binding sites. Since fibrinogen binding sites can be unmasked in the absence of ADP by the proteolytic enzyme chymotrypsin (Mustard et al., I979) and that chymotrypsin-treated platelets bind increased quantities of fibrinogen when stimulated with ADP compared to untreated platelets, it is tempting to speculate that in vivo platlet damage might result in the exposure of fibrinogen binding sites.

Platelet sensitivity to ADP-induced aggregation, as expressed by I/K_m, was increased in the diabetic patients. I/K_m is obtained from a kinetic analysis of the velocity of the primary wave of platelet aggregation in response to a range of ADP concentrations. Primary ADP-induced platelet aggregation is generally regarded as normal in diabetic subjects (Colwell et al., I978). This phase of aggregation is relatively difficult to quantify using a fixed low concentration of ADP, in view of the wide range of responses encountered. The technique described in this study uses a kinetic analysis of the aggregation curves produced by a range of ADP concentrations. By adjusting this range according to the responses obtained, platelets with widely different sensitivities to ADP may be compared. Since the result is not dependent on a single fixed concentration, pipetting errors may be minimized. A final advantage is that a series of aggregation velocities can be expressed as a single value, making comparisons between subjects simpler. Use of the Lineweaver-Burk plot, originally devized for analysis of enzyme kinetics, should not be taken to imply that platelet aggregation obeys Michaelis-Menton kinetics since it is likely that aggregation is considerably more complex. However, it would appear to be a useful method for the quantification of platelet aggregation.

Support for the view that enhanced aggregation is not confined to the second phase in diabetes is given by Porta et al. (I980) who found platelet shape change in response to ADP was increased in diabetes. If both shape change and primary aggregation are increased in diabetes, this argues for an abnormality in the aggregation response before the release reaction. It is therefore possible that the enhanced second phase reported by many authors, and enhanced platelet production of thromboxanes (Ziboh et al., I979; Halushka et al., I98I) could in part be secondary to this phenomenon. The report that when platelets from diabetic patients with retinopathy were incubated with dihomo-gamma-linoleic acid (a precursor of PGE_I which does not cause platelet aggregation) the quantity of MDA produced was not significantly different from control platelets (Zuzel et al., I979b) is also compatible with this hypothesis.

The present study found a strongly significant correlation between platelet aggregation (as expressed by I/K_m) and HbA_I. I/K_m was also higher in the diabetics with severe retinopathy compared with background changes only, but since the mean HbA_I was virtually identical in the two groups (II.2 $\pm$ 0.7 SEM and II.5 $\pm$ I.3% respectively), it seems possible that the relation between increased primary aggregation and diabetic control found in this study is independent of retinopathy. The relation between platelet function and metabolic control of diabetes will be discussed further in Part V of this Thesis.

Sensitivity of platelets from diabetic subjects was not found to be significantly correlated with the extent of fibrinogen binding. It has been recently been shown that ADP-induced platelet aggregation may be dissociated from fibrinogen binding. Whereas the platelets of type I Glanzmann's thrombasthenia do not aggregate in response to ADP and do not bind fibrinogen (Bennett & Vilaire, I979), the platelets of type II thrombasthenia do not aggregate with ADP but fibrinogen binding is only slightly subnormal (Lee et al., I98I). However, a more likely explanation is that the experimental conditions used for the study of platelet aggregation and fibrinogen binding were different : fibrinogen binding of washed platelets was measured under static conditions at room temperature at room temperature whereas aggregation was measured in stirred PRP at 37°C.

In conclusion, this study suggests that the platelet membrane may be altered in diabetic patients, as shown by increased

fibrinogen binding. This abnormality seemed to be related to the presence of severe retinopathy. Sensitivity of platelets to ADP was also found to be abnormal in diabetics, and appeared to be related to the quality of metabolic control.

PART IV PLATELETS AND PROSTAGLANDINS IN DIABETES MELLITUS

SECTION I PROSTACYCLIN PRODUCTION BY VASCULAR ENDOTHELIAL CELLS IN THE PRESENCE OF SERUM FROM DIABETIC AND CONTROL SUBJECTS

PROSTACYCLIN PRODUCTION BY VASCULAR ENDOTHELIAL CELLS CULTURED IN THE PRESENCE OF SERUM FROM DIABETIC AND CONTROL SUBJECTS

INTRODUCTION

As with other aspects of platelet behaviour in diabetes, there have been discordant findings when the thromboxane-prostacyclin system has been studied. Stuart et al (I979) reported that MDA (a by-product of the thromboxane pathway) production was increased in the platelets from pregnant diabetics compared to non-diabetics and Ziboh et al. (I979) and Halushka et al. (I98I) found that increased quantities of thromboxane B_2 were synthesized when PRP from diabetics was incubated with arachidonic acid, the latter authors reporting a positive correlation between the amount of thromboxane and the blood glucose at the time of study.

On the other hand, Davis et al. (I979) were unable to detect significant differences in the plasma levels of thromboxane B_2 between diabetics and controls and Tindall et al. (I98Ib) found that thrombin-stimulated PRP from diabetics produced significantly less MDA than the controls. The situation is further complicated by the suggestion that the half-life of thromboxane A_2 is longer in the plasma of diabetic subjects compared with controls (Lagarde et al., I980).

Similar disagreements are encountered when prostacyclin (PGI_2) production is considered. When PGI_2 production by segments of vein (Silberbauer et al., I979) or artery (Johnson et al., I979) from diabetics was measured by bioassay, PGI_2-like activity was reduced compared with biopsies from non-diabetic controls. However, Davis et al. (I98I)were unable to find significant differences between diabetics and controls, though there was a trend towards lower levels in diabetics with proliferative retinopathy. Plasma levels of 6-keto-$PGF_{I\alpha}$ (a stable metabolite of PGI_2) have been reported to be reduced (using gas-liquid chromatography) in diabetics with proliferative retinopathy (Dollery et al., I979), though when a radioimmunological technique was used, no significant differences were observed (Davis et al., I979, I98I).

Experiments using rats made diabetic with streptozotocin have shown that thromboxane B_2 production was slightly increased (Gerrard et al., I980) and PGI_2 production by vascular segments was reduced (Carreras et al., I980; Gerrard et al., I980; Harrison et al., I980)

Moreover, these abnormalities were reversed when the animals were treated with insulin (Harrison et al., I980) or pancreatic islet cell transplantation (Gerrard et al., I980), which suggests that poor diabetic control may be one factor contributing to reduced PGI_2 production.

In patients with severe and progressive proliferative retinopathy, hypophysectomy has been shown to slow the deterioration of vision (Lundbaek et al., I969). Such hypophysectomized patients do not appear to show the same platelet hyperaggregability as non-hypophysectomized patients with proliferative retinopathy (Passa et al., I974).

The aim of the present study was to test the hypothesis that serum from diabetic subjects with proliferative retinopathy contains factors which alter the synthesis or release of PGI_2 from the vascular endothelium, and to determine whether such factors are influenced by hypophysectomy.

MATERIALS AND METHODS

Subjects

Fifteen diabetics (I3 men, 2 women) were studied. All were insulin dependent diabetics with proliferative retinopathy confirmed by indirect ophthalmoscopy by an experienced ophthalmologist. Clinical details of the patients are shown in Table I5. Mean age at the time of study was 48.8 years (range 33 - 63 years), with a mean time since diagnosis of 24.2 years (0 - 4I years). Five patients had previously been surgically hypophysectomized and repeated pituitary function tests following surgery confirmed absence of anterior pituitary function. All were receiving hormone replacement therapy with cortisol and thyroxine at the time of study.

Fifteen healthy non-diabetic controls (I3 men, 2 women) were chosen from the medical staff. The mean age was 28.7 years (range 22 - 56 years). Smoking habits were similar to the diabetics (four smoked I0 - 20 and four 5 - I0 cigarettes/day). The controls were matched for sex with the diabetics. None of the subjects were taking medications known to influence platelet function or prostaglandin production for at least two weeks prior to the tests.

Preparation of Human Serum

Two-hr post-prandial venous blood samples were taken into

two 50ml sterile glass tubes. The blood was incubated at 37°C for 2hr, then centrifuged at 2,000 g for 20 min at room temperature. The resulting serum was filtered through first 0.45u then 0.22u filters (Millipore, Bedford, Massachusetts) and stored in sterile glass containers at 4°C until required.

Endothelial Cell Culture

Materials

Foetal Calf Serum was obtained from Biomérieux (Marcy l'Etoile, France) and was decomplemented by warming at 56°C for 30 min. Thereafter, it was stored at 4°C until use.

Cord Buffer was made by dissolving 80g sodium chloride, I.4g disodium hydrogen phosphate (7 H_2O), 3g potassium chloride, 20g glucose and 0.2g potassium dihydrogen phosphate in Il distilled water. This resulted in a buffer of pH 6.5 which was filtered through 0.22u Millipore filters (Millipore, Molsheim, France) and stored in aliquots at - 20°C until required. Before use, Colomycin-Penicillin (Roger Bellon, Neuilly, France) 6,000U/ml was added.

Collagenase (type CLS 4I96) was obtained from Mallet Chemicals (Roissy, France). 0.4g of the crystalline powder was added to I00ml cord buffer at 4°C and the mixture stirred at 4°C for I0 min to obtain dissolution of the collagenase. The solution was then made up to 200ml with cord buffer, giving a final concentration of 0.2%. The solution was then centrifuged at 3,000 g for 30 min at 4°C, the pH adjusted to 7.4 with 0.IN sodium hydroxide and passed through first 0.8u and then 0.22u Millipore filters. It was then stored in aliquots at -20°C until required.

Culture Buffer was prepared by adding to medium MI99 (Biomérieux) the following products : penicillin I00U/ml, streptomycin I00mg/ml, Fungizone 2.5mg/ml, HEPES (hydroxy-2-ethyl-piperazinyl-I-2-ethane-sulphonic acid) I5mM, L-glutamine 2mM and I3mM sodium bicarbonate (all from Flobio, Paris). The solution was filtered through 0.22u Millipore and stored at4°C.

Methods

Culture of Endothelial Cells was performed according to the method of Jaffe et al. (I973a). Shortly after birth, the umbilical cord was severed from the placenta and placed in a sterile container filled with 50ml cord buffer. The container was kept at 4°C until required. Cords which had been traumatized, or that were less than I5cm were discarded.

Thereafter, the umbilical cord was inspected and clamp marks at each end of the cord were cut off. The umbilical vein was cannulated and rinsed two times with cord buffer. The vein was then filled with collagenase solution, the two ends secured by clamping the plastic cannulae, and the cord immersed in 0.I5M saline at 37°C for 6-8 min, keeping the ends out of the saline. The cord was then gently massaged to complete detachment of the endothelial cells and the vein was drained into a sterile plastic 50ml centrifuge tube and the vein was rinsed with 30ml cord buffer. Each tube contained I0ml MI99 with 20% foetal calf serum to neutralize the remaining collagenase. The cells were centrifuged at 250 g for I0 min, the supernatant was discarded and the cells resuspended in the culture buffer with 20% foetal calf serum. After a further centrifugation the cells were resuspended in culture buffer with 20% calf serum. A cell count was made using a Neubauer counting chamber and the count was adjusted to I00,000/ml. The cell suspension was then added to sterile 60mm diameter plastic Petri dishes (Falcon Plastics, Oxnard, California) at a concentration of 40,000/cm^2. All manipulations were performed under a sterile hood. The cultures were incubated at 37°C and in an atmosphere of 5% carbon dioxide, using an incubator produced by Forma Scientific (Marietta, Ohio). After 24 h , the culture was removed and replaced with fresh culture medium, 20% human serum replacing the foetal calf serum (implantation of the endothelial cells on the plastic dishes was found to be less efficient when human serum was used). The culture medium was changed every 48 hr until the endothelial cells reached confluence (7 - 9 days). Cultures were assessed for purity initially by transmission electron microscopy (endothelial cells being recognized by their morphological characteristics and by the presence of Weibel-Palade bodies; Jaffe et al., I973a) and routinely by phase contrast microscopy. Cultures which did not reach confluence by nine days, and those which were contaminated with fibroblasts were discarded.

Stimulation of Endothelial Cells

When the cells had reached confluence, thrombin-stimulated release of PGI_2 was performed using a modification of the method of Weksler et al. (I978). The cultures were gently rinsed with two volumes of buffer A (sodium chloride I50mM, potassium chloride 5mM, calcium chloride I.8mM, magnesium chloride ImM, glucose 5mM and HEPES I0mM, pH 7.6). One ml buffer A containing 0.I NIHu/ml bovine thrombin was then added to the cultures. (Roche bovine thrombin 5,000 NIHu was dissolved in 5ml imidazole buffered saline (0.04M, pH 7.4) and the solution was added to 5ml glycerol, giving a final concentration of 500 NIHu/ml. The thrombin was divided into small aliquots and stored at - 20°C until use. A fresh dilution of thrombin was prepared for

each experiment) The Petri dish was then placed on a rotating horizontal platform (I00 rpm) for exactly 2 min. The supernatant was then removed and placed in a plastic tube standing in an ice bath. All manipulations were performed at room temperature. After measurement of PGI_2-like activity, the remaining supernatant was frozen at - 20°C until required for the measurement of 6-keto-$PGF_{I\alpha}$ concentrations. The endothelial cells were detached from the Petri dishes at the end of the experiment by incubation with cord buffer containing 0.02% EDTA (Biomérieux) for 20 min and counted using a Neubauer chamber.

PGI_2-like Activity

PGI_2-like activity was measured by the inhibitory effect of the supernatant on ADP-induced platelet aggregation. Non-fasting venous blood (the same donor was used for all experiments) was taken into trisodium citrate to give a final citrate concentration of 0.I29M. The platelet count of the PRP was measured by the method of Brecher and Cronkite (I950) and the platelet count was adjusted to 250 - 300 x $I0^9/l$ with PPP. Platelet aggregation was performed using a Labintec aggregometer (Marseille, France), with a stirring speed of I,I00rpm. 0.Iml of supernatant was added to 0.4ml PRP in the aggregometer cuvette and incubated without stirring for 45 s at 37°C. The PRP was then stirred for I5 s before 2uM ADP (final concentration) was added. The chart speed was 25.2cm/min. The initial rate of aggregation was measured by drawing a tangent through the steepest part of the aggregation curve and was expressed as cm/min (the sensitivity of the recorder having been set so that PRP and PPP corresponded to I0% and 90% respectively). PGI_2-like activity was expressed as a percentage of the inhibition of the initial rate of aggregation compared with a control aggegation curve using 0.4ml PRP and 0.Iml buffer A containing 0.I NIHu/ml thrombin. Since PGI_2 is labile even at 0°C, care was taken to complete the estimation of PGI_2-like activity within 5 min of stimulating the endothelial cells with thrombin, though preliminary experiments showed that activity remained stable on ice for up to I0 min.

6-Keto-$PGF_{I\alpha}$

The frozen supernatants were incubated at 37°C for I5 min to ensure complete conversion of PGI_2 to 6-keto-$PGF_{I\alpha}$. The concentration of 6-keto-$PGF_{I\alpha}$ in the supernatant was measured in duplicate by radioimmunoassay, using the kit supplied by New England Nuclear (Boston, Massachussets). Results were expressed as the quantity of 6-keto-$PGF_{I\alpha}$ produced in ng/Petri dish.

Ideal Weight was calculated using the Metropolitan Life Assurance Co. tables (Documenta Geigy, 1970).

Haemoglobin A_I was measured using the microcolumn kit produced by Biorad laboratories (Richmond, California) by the Department of Clinical Chemistry at Hôpital St Louis.

Serum Creatinine was measured by autoanalyzer.

Statistics

Results between diabetics and controls were compared using Wilcoxon's rank sign test for paired samples. Correlations were calculated using Kendall's rank sign test.

RESULTS

No difference was observed in the time that cells cultured in diabetic serum reached confluence compared with those grown in control serum, though cells obtained from different umbilical cords reached confluence between seven to nine days. At confluence, the cultures were indistinguishable on phase-contrast microscopy. A typical endothelial cell monolayer is shown in Plate I. Moreover, the number of cells/culture dish was similar in endothelial cells grown in the presence of diabetic or control serum (Table I6).

As shown in Fig. 2I, the PGI_2-like activity produced by endothelial cells cultured in the presence of sera from the diabetic subjects was significantly less than that of cells cultured in control sera (mean $\pm$ SEM 2I.9 $\pm$ 4.8 compared with 28.3 $\pm$ 5.I%; $p < 0.05$). Similarly, 6-keto-$PGF_{I\alpha}$ concentrations were significantly lower in cells grown in diabetic compared with control sera (3.I5 $\pm$ 0.68ng/dish compared with 3.94 $\pm$ 0.9Ing/dish; $p < 0.05$).

The correlation between the concentration of 6-keto-$PGF_{I\alpha}$ and PGI_2-like activity for each sample of supernatant is shown in Fig. 22, the correlation being statistically significant (τ 0.52; $p < 0.00I$). It may also be seen that the correlation between bioassay and radioimmunoassay was closer in supernatants derived from endothelial cells grown in diabetic compared with control serum. As shown in Fig. 22, the range of PGI_2-like activities and 6-keto-$PGF_{I\alpha}$ concentrations was wide. For this reason, results were also expressed as a ratio :

PGI_2 production (PGI_2-like activity or 6-keto-$PGF_{I\alpha}$ concentration) by endo-

thelial cells cultured in diabetic serum divided by PGI_2 production of the cells grown in the corresponding control serum. The ratios for the I5 pairs of sera are shown in Fig. 23.

When the rank of the 6-keto-$PGF_{I\alpha}$ ratios for the I5 diabetics was correlated with the rank for the PGI_2-like activity, the relationship was non-significant, though when only the I0 diabetics who had not been hypophysectomized were considered, a significant correlation was found (τ 0.5I; $p < 0.05$).

There was a statistically significant relationship between the 6-keto-$PGF_{I\alpha}$ ratios and the concentration of HbA_I, a higher HbA_I being associated with a lower production of 6-keto-$PGF_{I\alpha}$ (τ 0.49; $p < 0.05$), as shown in Fig. 24. There were no significant correlations between the 6-keto-$PGF_{I\alpha}$ ratios and age, duration of diabetes or serum creatinine levels, and the ratios were not different when hypophysectomized were compared with non-hypophysectomized diabetics (Fig. 23).

DISCUSSION

The results of this study indicate that when human endothelial cells were cultured in the presence of serum of diabetics with proliferative retinopathy, thrombin-induced release of PGI_2 was significantly diminished compared with cells grown in control serum. Moreover, this reduced capacity to generate PGI_2 appeared to be related to the degree of diabetic control, as assessed by the concentration of glycosylated haemoglobin.

This phenomenon seems unlikely to be accounted for by a non-specific effect on cellular growth or proliferation, since cells cultured in diabetic serum had the same morphology on light microscopy, reached confluence at the same time and had similar cell counts at confluence compared with cells grown in control serum.

It is possible that substances other than PGI_2 with platelet-inhibitory properties are released from endothelial cells. A significant correlation was found between PGI_2-like activity and concentrations of 6-keto-$PGF_{I\alpha}$, suggesting that the platelet-inhibiting properties of the supernatant were parallelled by the concentration of 6-keto-$PGF_{I\alpha}$ in the more specific radioimmunoassay.

Thrombin-induced PGI_2 release is dose-related and time-dependent (Czervionke et al., I979). The concentration of thrombin

(0.I NIHu/ml) and the time of incubation (2 min) used in the present study were chosen as a compromise, since preliminary experiments showed that higher concentrations of thrombin in buffer A induced aggregation when added to PRP, thus partially masking the inhibitory effect of PGI_2, while longer incubation times resulted in loss of PGI_2 -like activity.

Differences in PGI_2 production between cells grown in diabetic compared with controls though statistically significant, were relatively small. An important limitation of the method is that to avoid cytotoxicity, the proportion of serum that can be added to the culture should not exceed 20% so that any possible inhibitory factors in the serum are diluted by I : 5. In addition, as can be observed in Fig. 22, there was a considerable range of both PGI_2-like activity and 6-keto-$PGF_{I\alpha}$ concentrations produced by endothelial cells cultured in both diabetic and control serum. This variation in PGI_2 production in endothelial cells obtained from different umbilical cords cultured under the same conditions has been previously observed by others (Baenzinger et al., I979); the cause of this variability is unknown. As Gerrard et al. (I980) have pointed out, even a relatively minor shift in the balance between proaggregatory thromboxane and inhibitory PGI_2 production could increase the risk of thrombosis.

The finding of reduced PGI_2 production by human endothelial cells is compatible with animal studies which showed that reduced vessel wall production of PGI_2 in diabetic rats was reversible by insulin injections or islet cell transplantation (Harrison et al., I980; Gerrard et al., I980). These studies suggest that metabolic factors may be involved. Since all the patients in the present study had proliferative retinopathy, it is possible that their serum contained inhibitory factors found only in patients with proliferative retinopathy. However, the finding of a significant correlation between the reduction in 6-keto-$PGF_{I\alpha}$ concentration and HbA_I argues for glycaemic control being of importance in the regulation of PGI_2 production. The effect of glucose on the production of 6-keto-$PGF_{I\alpha}$ concentrations produced by thrombin-stimulated endothelial cells will be considered in the following Section.

There were no obvious differences in cellular growth, morphology, or PGI_2 production by endothelial cells cultured in serum from hypophysectomized compared with non-hypophysectomized diabetics. The growth factor obtained from bovine pituitary glands seems not to be essential for the growth of normal endothelial cells in culture

(Gajdusek et al., I980). However, if this factor is absent during several sub-cultures, the morphology and function of the cells are modified (Vlodavsky et al., I979). The present study used primary cultures only, and the results suggest that absent pituitary function does not influence endothelial cell growth under these conditions.

In conclusion, the results of this study show that PGI_2 production from thrombin-stimulated endothelial cells grown in serum from diabetics with proliferative retinopathy is reduced compared with cells cultured in non-diabetic serum. This inhibition seemed to be related to the degree of glycaemic control of the diabetes.

SECTION 2 INFLUENCE OF GLUCOSE CONCENTRATION ON PGI_2 PRODUCTION BY CULTURED VASCULAR ENDOTHELIAL CELLS

INFLUENCE OF GLUCOSE CONCENTRATION ON PGI_2 PRODUCTION BY CULTURED VASCULAR ENDOTHELIAL CELLS.

INTRODUCTION

The results obtained in the previous Section suggest a possible relationship between PGI_2 production and the degree of glycaemic control of the diabetic serum. That glucose can influence prostaglandin synthesis was shown by Tannerbaum et al. (1979) who reported that glucose inhibited the production of PGE_2 and $PGF_{2\alpha}$ by the isolated rat renal papilla preparation in a dose-related manner.

The aim of the present study was to assess the effect of different concentrations of glucose in the culture medium on PGI_2 production by endothelial cells. In view of the good correlation between PGI_2-like activity and concentration of 6-keto-$PGF_{I\alpha}$ reported in the previous Section, 6-keto-$PGF_{I\alpha}$ only was measured in these experiments.

METHODS

Endothelial cells derived from human umbilical veins of non-diabetic women were cultured as described in the preceeding Chapter, though in the present experiments, 35mm diameter Petri dishes were used. The culture medium was MI99 with antibiotics and 20% human serum as previously described. The same batch of serum from a non-diabetic subject was used throughout these experiments. Immediately before use, the culture medium was supplemented with a range of concentrations of glucose : 5.5mM (no extra glucose added), II.0mM, I6.5mM, 22mM, 27.5mM and 38.5mM (final concentrations).

The culture medium of each culture was changed every 48h until the cultures reached confluence (7-9 days), when thrombin-stimulated PGI_2 release was performed (0.I NIH u/ml) as described in the preceeding Section. Two different protocols were performed :

Protocol A

Endothelial cells from a single umbilical cord were seeded on six 35mm diameter Petri dishes. After implantation with MI99 containing 20% foetal calf serum for 24h, the culture medium was changed to that containing 20% human serum containing the six glucose concentrations as described above. At confluence, the cultures were rinsed twice with buffer A, then 0.5ml buffer A with 0.I NIHu/ml thrombin was added, cultures being agitated for 2 min. The supernatant was then removed and

immediately frozen at - 20°C until measurements of 6-keto-$PGF_{I\alpha}$ concentrations were performed. Three separate experiments using the endothelial cells from three different umbilical cords were carried out.

Protocol B

The endothelial cells from a single umbilical cord were implanted into ten 35mm diameter Petri dishes using MI99 and foetal calf serum as described above. After 24h, the culture medium was changed to MI99 containing 20% human serum. Five dishes were cultured in a medium containing 5.5mM glucose, the remainder were cultured with a glucose concentration of 22.0mmol/l. When the cells reached confluence, thrombin-induced PGI_2 release was performed as described in protocol A, but with incubation times of 30s , I min, 2 min, 5 min and IO min. For each incubation, one dish cultured in 5.5mM glucose and in the other, 22mM glucose were tested. The supernatants were stored at - 20°C until required. Three experiments using endothelial cells from three different umbilical cords were performed.

6-keto-$PGF_{I\alpha}$ concentrations

The concentration of 6-keto-$PGF_{I\alpha}$ of each supernatant was measured in duplicate using the radioimmunoassy described in the previous Section.

RESULTS

Protocol A

There was no difference in the light microscopic appearance, or in the time taken to reach confluence, of endothelial cells grown in glucose-supplemented media. However, at the highest concentration of glucose tested (38.5mM) there was a tendency for cells to become detached when they reached confluence. The results of the concentrations of 6-keto-$PGF_{I\alpha}$ produced by the thrombin-stimulated cells are shown in Table I7. There were no consistent differences between cells cultured in the various glucose-supplemented media, though there was a tendency to increased levels from cells cultured in media containing 38.5mM glucose.

Protocol B

As for Protocol B, there were no differences in morphology or time taken to reach confluence between endothelial cells cultured in media containing 5.5 and 22mM glucose. The results are shown in Table I8 and the means from the three different cell cultures are presented in Fig. 25.

At both concentrations of glucose, there was a time-dependent increase in the production of 6-keto-$PGF_{I\alpha}$. Howevever, there were no consistent differences between endothelial cells grown in medium containing 5.5mM and 22mM glucose.

DISCUSSION

These results indicate that raised glucose concentrations do not appear to directly inhibit PGI_2 production by thrombin-stimulated endothelial cells. Indeed, at 38.5mmol/l glucose, there was a tendency to increased PGI_2 release. This stimulatory effect may have been due to cell injury secondary to the increased osmolarity of the culture medium. Toxicity at this glucose concentration is also suggested by instability of cell attachment to the Petri dish. Concentrations of plasma glucose over 40mmol/l are frequently encountered during diabetic ketoacidosis and non-ketotic hyperosmolar states (see Part V, Section I). The results of the present in vitro study suggest that such high glucose concentrations are directly toxic to the endothelial cells, and could be one factor causing endothelial damage in vivo.

The inhibitory factor described in the preceeding may have been caused by glycosylated serum proteins, rather than glucose directly. This appears to be unlikely, since in vitro glycosylation of proteins such as albumin proceeds rapidly (Dolhoffer & Wieland, 1979) at 37°C, making it probable that a proportion of serum proteins were glycosylated in the culture medium during incubation with the endothelial cells. Certain non-esterified fatty acids such as linoleic acid have been shown to be potent inhibitors of PGI_2 synthesis when added to endothelial cell cultures (Spector et al., 1980). Non-esterified fatty acids were not measured in the present study. Though total amounts of these fatty acids are increased in the serum of insulin-dependent diabetics, there appears to be no major alteration in the relative concentrations of individual fatty acids (Schrade et al., 1963) which could account for the results observed in the preceeding Section.

SECTION 3 EFFECT OF SERUM FROM WELL AND POORLY-CONTROLLED DIABETICS ON PGI_2 PRODUCTION BY CULTURED VASCULAR ENDOTHELIAL CELLS

EFFECT OF SERUM FROM WELL AND POORLY-CONTROLLED DIABETICS ON PGI_2 PRODUCTION BY CULTURED VASCULAR ENDOTHELIAL CELLS

INTRODUCTION

In the preceeding Section, it was concluded that a high glucose concentration was not the factor responsible for the reduction in PGI_2 production by endothelial cells cultured in serum from diabetics with proliferative retinopathy. The experiments described in this Section were designed to measure 6-keto-$PGF_{I\alpha}$ production by endothelial cells cultured in the sera from well and poorly-controlled diabetics without vascular complications.

MATERIALS AND METHODS

Subjects

Sixteen male insulin-dependent diabetics were selected for the study. None had symptoms or signs of macro or microvascular disease. Eight were considered to have good glycaemic control and eight had poor control of their diabetes. Good control was defined as an HbA_I at the time of venesection of less than 8.5% (upper limit of normal in this laboratory is less than 7.5%) and poor control was defined as an HbA_I of I2.0% or more. Diabetics over the age of 50 years, and those taking drugs other than insulin were not included in the study.

After informed consent, non-fasting venous blood was removed from the patients. Serum was prepared as described in Section I of this Part. The filtered serum was stored at 4°C until required in sterile glass containers. All sera were used within 3 weeks.

Endothelial Cell Culture and 6-keto-$PGF_{I\alpha}$ production

Endothelial cells derived from the umbilical vein of non-diabetic women were cultured as described in the previous Sections. After implantation in 35mm diameter plastic Petri dishes using MI99 containing 20% foetal calf serum for 24h, the medium was replaced with MI99 with 20% diabetic serum. For each experiment, two Petri dishes contained endothelial cells cultured in serum from a poorly-controlled diabetic, and two contained cells cultured in serum from a well-controlled diabetic. The pairs of diabetics were otherwise matched as closely as possible for age and duration of diabetes.

When the cells reached confluence, the cultures were

rinsed twice and stimulated with 0.5ml buffer A containing 0.I NIHu/ml bovine thrombin as described in Section I. After 2 min agitation, the supernatant was removed and stored at - 20°C until required.

6-keto-$PGF_{I\alpha}$

Each supernatant was assayed in duplicate for 6-keto-$PGF_{I\alpha}$ concentration, using the radioimmunoassay provided by New England Nuclear (Boston). For each diabetic serum, results are expressed as the mean 6-keto-$PGF_{I\alpha}$ production (ng/culture dish) by duplicate endothelial cultures.

Haemoglobin A_I and Blood Glucose

HbA_I concentrations (microcolumn technique, Biorad laboratories) and the blood glucose (glucose oxidase technique) were measured at the same time that blood was removed to obtain serum.

Statistics

Comparison of the results between groups was made using Wilcoxon's rank sign test for paired samples, and correlations were made with Kendall's rank correlation test.

RESULTS

Details of the eight pairs of diabetics are shown in Table I9. Well and poorly diabetics were closely matched for age and duration of diabetes. In addition, per cent ideal weight (calculated from Metropolitan Life Assurance Tables, Documenta Geigy, I970) was similar in well-controlled (mean I00.I, range 86-II4%) and poorly controlled (mean 96.0, tange 86-I07%) diabetics.

As shown in Table I9, both post-prandial blood glucose and HbA_I concentrations were significantly higher in poorly controlled compared with well-controlled diabetics ($p < 0.0I$).

Results of 6-keto-$PGF_{I\alpha}$ production by endothelial cells cultured in serum from well-controlled and poorly-controlled diabetics are shown in Fig. 26. Though the mean value of 6-keto-$PGF_{I\alpha}$ was slightly higher in the group of well-controlled compared with the poorly-controlled group (0.77ng/dish compared with 0.69ng/dish) this difference was not statistically significant. There was no difference either in the cell morphology or in the time the cells took to reach confluence between endothelial cells cultured in serum from well or poorly controlled diabetics.

When 6-keto-$PGF_{I\alpha}$ production was related to the duration of diabetes, irrespective of the quality of diabetic control, the highest values of 6-keto-$PGF_{I\alpha}$ tended to be produced by cells cultured in serum from those diabetics with the shortest duration of diabetes (Fig.27). Using Kendall's rank correlation test, this correlation did not reach statistical significance (τ - 0.275, $p > 0.05$). However, when the diabetics were arbitrarily divided into two groups on the basis of duration of diabetes, cells cultured in serum from those diabetics with a duration of diabetes less than eight years had significantly higher 6-keto-$PGF_{I\alpha}$ production ($p < 0.05$) compared with those sera from diabetics with a duration greter than eight years (Fig. 28).

DISCUSSION

These results show that PGI_2 production by endothelial cells cultured in the serum from poorly-controlled diabetics did not differ significantly from that produced by cells grown in serum from well-controlled patients. However, an inverse relationship between 6-keto-$PGF_{I\alpha}$ concentration and the duration of diabetes was found. It is therefore possible that severe vascular complications require to be present before the depressor effect of diabetic serum on PGI_2 production is observed, the metabolic control of the diabetes having a relatively minor effect. Since the extent and severity of vascular complications correlate with the duration of diabetes (Pirart, I978; Jarrett & Keen, I979), it may be that patients described in this Section with the longest duration of diabetes had subclinical vascular disease, resulting in sera which depressed PGI_2 production.

The nature of this factor remains to be determined. Since serum was used for all the endothelial cell cultures described in this Thesis, it is possible that an inhibitor of PGI_2 production is released from platelets, or formed during blood coagulation during preparation of the serum. In this regard, β-thromboglobulin has been shown to bind to bovine endothelial cells in culture and to inhibit PGI_2 synthesis of these cells (Hope et al., I979). Though the plasma concentrations of β-thromboglobulin are frequently increased in diabetics, particularly in those with microvascular complications (Burrows et al., I978; Preston et al., I978) it is not known whether serum concentrations of β-thromboglobulin are higher in diabetics compared with controls. Further experiments are required to establish whether endothelial cells cultured in a medium

supplemented with platelet-poor plasma (rather than serum) from diabetics with proliferative retinopathy is reduced compared with cells grown in a medium containing control plasma.

In summary, the results from these three Sections indicate that PGI_2 production by endothelial cells cultured in the presence of sera from patients with proliferative retinopathy is significantly reduced compared with cells grown in control serum. This depressor effect appeared to be related to the quality of metabolic control of the diabetes. The effect was not reproduced by growing the endothelial cells in a glucose-enriched medium, nor when a group of poorly-controlled diabetics without overt vascular complications were compared with a group of well-controlled diabetics, also without clinically detectable vascular disease.

PART V METABOLIC CONTROL OF DIABETES AND HAEMOSTATIC FUNCTION

METABOLIC CONTROL OF DIABETES AND HAEMOSTATIC FUNCTION

In view of the large numbers of publications devoted to aspects of the haemostatic function in diabetic patients, it is perhaps surprising that so few studies have been concerned with the effect of the metabolic disturbances of diabetes on haemostasis.

This Section describes two studies relating to this problem. The first involves the disturbances of acute metabolic decompensation - diabetic coma and the second deals with the sequential changes in certain components of the haemostatic system during the establishment of glycaemic control in patients with newly-diagnosed non-insulin dependent diabetes.

SECTION I HAEMOSTATIC CHANGES IN DIABETIC COMA

HAEMOSTATIC CHANGES IN DIABETIC COMA

INTRODUCTION

Despite modern intensive therapy, acute metabolic decompensation remains a potentially fatal complication of diabetes mellitus. Estimates of mortality rates due to diabetic ketoacidosis range from 0.5 to as high as I5%, depending on the centre, and rates do not seem to have altered greatly over the past 20 years (Hockaday & Alberti, I972; Clements & Vourganti, I978). In the non-ketotic hyperosmolar variety of diabetic coma, even higher mortality rates of I5 - 20% are generally recorded (Gordon & Kabadi, I976).

Arterial thrombosis may now be responsible for up to one third of deaths from diabetic ketoacidosis (Clements & Vourganti, I978) and is a frequent complication in non-ketotic hyperosmolar coma (Whelton et al., I97I). In addition, Timperley et al. (I974) have shown that localized deposition of fibrin may occur in the cerebral capillaries of fatal cases of ketoacidosis. Over the past decade, a number of investigators have described the association of disseminated intravascular coagulation and diabetic coma (Table 20). Most of the described cases have been ketoacidotic, but two patients probably had non-ketotic hyperosmolar comas. The diagnosis of disseminated intravascular coagulation was based on haematological evidence in nine, but pathological evidence of fibrin deposition was described in six cases.

Such case reports have been sporadic, and laboratory studies were only undertaken after clinical signs had become apparent. A prospective study was therefore designed to determine the nature of hamostatic changes in unselected cases of diabetic ketoacidosis and the hyperosmolar syndrome.

MATERIALS AND METHODS

Patients

Fifteen diabetics, admitted to the Aberdeen Teaching Hospitals with ketoacidosis or the hyperosmolar syndrome over a period of I2 months, were included in the study. Diabetic ketoacidosis was arbitrarily defined as a plasma glucose greater than I4mmol/l and a serum bicarbonate of IImmol/l or less in association with ketonuria. The non-ketotic hyperosmolar syndrome was defined as a plasma glucose

greater than 30mmol/l with a serum osmolarity of more than 350mOsmol/l (Gordon & Kabadi, 1976), serum bicarbonate greater than 20mmol/l and with no more than a trace of ketonuria.

Where possible, a second "convalescent" sample of blood was obtained from the patients at a subsequent out-patient visit when the diabetic state was stable, the non-fasting plasma glucose was less than 10mmol/l and no detectable ketones were present in the urine.

Methods

Venous blood was removed from the patients as soon as possible after arrival at hospital (mean 2.2h, range 0.1 - 5h) for biochemical and haematological studies. Blood for assays of the coagulation system was immediately placed on ice for transport to the laboratory where it was immediately centrifuged at 1,500 g and 4°C within 30min of venepuncture.

Partial Thromboplastin Time, Prothrombin time were measured on fresh plasma, using the techniques described in Part II, Section 2.

Fibrinogen and Plasminogen were assayed in oxalated plasmas as described in Part II, Section 2. Plasmas were stored at - 70°C until required.

Factors V and VIII:C were measured on citrated samples stored at - 70°C as described in Part II, Section 2.

Whole Blood Platelet Count was performed using the method described in Part II, Section 1.

Antithrombin III was measured using the method described in Part II, Section 3.

Fibrin Degradation Products were estimated using the kit supplied by Wellcome Reagents (Beckenham, Kent).

Factor VIII-Related Antigen (VIIIR:Ag) was measured by immunoelectrophoresis using monospecific antibody to factor VIII produced by Behringwerke AG, Marburg, W. Germany, by the method described by Laurell (1966). Results were expressed as a percentage of the values obtained from the normal plasma pool described in Part II, Section 2.

Haematocrit was measured using a microhaematocrit centrifuge (Hawksley & Sons, Lancing Sussex).

Plasma Glucose was measured by the glucose oxidase method.

Serum Urea and Electrolytes were measured by autoanalyzer.

Urinary Ketones were measured by reagent strips (Ketostix, Ames Co.)

Serum Osmolarity was calculated using the formula :

$$\text{serum osmolarity (mOsmol/l)} = 2(Na + K) + G + U$$

where Na is the serum sodium, K the potassium, U the urea concentration and G the plasma glucose concentration in mmol/l (Gordon & Kabadi, I976).

Depth of Coma on admission was assessed clinically using a score of I - 4 (I = normal; 2 = drowsy, but responding to verbal commands ; 3 = responding only to painful stimuli; 4 = unrousable).

Correction for Dehydration Since dehydration occurs in most cases of diabetic coma, where possible, concentrations of haemostatic factors were adjusted for changes in haematocrit (Bennett et al., I967) :

$$\text{corrected concentration} = \text{measured concentration} \times \frac{H_I\ (100 - H_2)}{H_2\ (100 - H_I)}$$

where H_I is the haematocrit after coma and H_2 the haematocrit during coma. This formula is based on the assumption that the red cell mass remains relatively constant during dehydration, and that changes in haematocrit therefore reflect changes in plasma volume. Results are presented as both measured and corrected concentrations. The coagulation tests where an activity rather than a concentration are measured (e.g. prothrombin time) were left uncorrected.

Statistics

Comparison between the results before and after diabetic coma was made using the Wilcoxon rank sign test for paired samples.

RESULTS

Fifteen diabetics fulfilled the entry criteria and were admitted to the study. The diagnosis of diabetes was made for the first time in six patients on entry to hospital. Clinical details are shown in Table 2I and the biochemical data in Table 22. Findings in the I2 patients with ketoacidosis are presented separately from the three patients with the hyperosmolar syndrome.

Patients with Ketoacidosis

Patient No. 3 died 3h after admission following a cardiac arrest. The results of the haemostatic tests of the surviving II diabetics are summarized in Table 23. The normal values for fibrinogen, plasminogen, and antithrombin III shown in Tables 23 and 24 are those of the 34 non-diabetic controls previously discussed in Part II, Section I. Compared with convalescent values, platelet count, factor VIII:C factor VIIIR:Ag and fibrinogen degradation products were significantly increased. These differences were particularly striking in the case of factor VIII:C and VIIIR:Ag (Fig. 29). Antithrombin III concentrations were significantly lower and there was a significant shortening of the partial thromboplastin time during ketoacidosis compared with convalescent values (Table 22). Factor V, fibrinogen, plasminogen and prothrombin time were not significantly different from convalescent values.

When the results were corrected for the effect of dehydration using the formula described above, factors VIII:C and VIIIR:Ag were still significantly higher and antithrombin III concentrations lower, during ketoacidosis. The differences in platelet count and fibrin degradation products were no longer significant, though seven out of the eleven patients had corrected concentrations of fibrin degradation products greater than IOug/ml compared to only one out of eleven convalescent samples. In addition, corrected mean plasminogen concentrations were significantly lower during ketoacidosis.

The mean $\pm$ SEM factor VIIIR:Ag : VIII:C ratio was I.44 $\pm$ 0.27 during ketoacidosis , which was significantly higher than the convalescent value of 0.73 $\pm$ 0.08 ($p < 0.01$). There was no significant correlation between the levels of the various haemostatic factors and the depth of coma, the presence of overt infection, plasma glucose, serum bicarbonate and the serum osmolarity. Neither the two patients with severe hypokalaemia (Nos. 2, II) nor the two with a serum urea greater than 30mmol/l

had haemostatic values strikingly different from the remaining diabetic patients.

Patients with the Hyperosmolar Syndrome

As can be seen from Table 2I, the three patients with the hyperosmolar syndrome (Nos. I3, I4, I5) comprized the most elderly patients in the study. Two died in the course of their admission to hospital (Nos. I4, I5). The haemostatic results on admission are shown in Table 24, together with the fatal case with ketoacidosis (No. 3). Results are necessarily presented as uncorrected values. As can be seen from Tables 22 and 23, patients 3 and I3 showed results similar to the surviving patients with ketoacidosis. In contrast, the two fatal hyperosmolar cases (Nos. I4, I5) had thrombocytopenia, low concentrations of plasminogen and high levels of fibrin degradation products.

Patient I4 had a past history of ischaemic heart disease and intermittent claudication. On admission, he was found to have impalpable pulses from the left femoral downwards. The hyperosmolar state was rapidly corrected with fluid and insulin therapy, but perfusion of the left leg did not improve, below-knee amputation being required six days later, followed by death after a further eight days. The severe thrombocytopenia persisted throughout his final illness. Permission for post-mortem examination was refused.

Patient I5 was admitted in hyperosmolar coma. On examination, she had minimal abdominal tenderness and absent bowel sounds. Death occurred I2h after admission. At autopsy, she was found to have 80cm of infarcted small intestine and when sections were stained with Martius Yellow, Crystal Scarlet and Soluble Blue, there was evidence of fibrin deposition in capillaries, arterioles and venules of cerebral cortex (Plate 2) , kidney (Plate 3), lung and small intestine.

DISCUSSION

This study has shown that characteristic changes occur in the haemostatic system during diabetic ketoacidosis, the most striking being a rise in factor VIII:C and factorVIIIR:Ag. Raised factor VIII levels are not exclusive to diabetic ketoacidosis, increases having been described in a variety of inflammatory, thromboembolic and neoplastic conditions (Holmberg & Nilsson, I974). Raised concentrations of factor

VIIIR:Ag (Pandolfi et al., I974; Coller et al., I978; Lufkin et al., I979; Gensini et al., I979) and factor VIII:ristocetin activity (Bensoussan et al., I975; Coller et al., I978; Lufkin et al., I979; Gensini et al., I979) have frequently been observed in stable diabetes. Raised levels were only found in patients with proliferative retinopathy in certain studies (Bensoussan et al., I975; Coller et al., I978), but others have reported high levels in patients free from vascular complications (Pandolfi et al., I974; Lufkin et al., I979; Gensini et al., I979). Apart from Coller et al. (I978), who found no correlation between HbA_I and concentrations of factor VIIIR:Ag, none of these studies attempted to determine whether there was a relation between factor VIIIR:Ag and diabetic control, though Gonzalez et al. (I980) found that in insulin-treated diabetics strictly controlled under hospital conditions, improvement in diabetic control was parallelled in some cases by a reduction in factor VIII: ristocetin cofactor activity. Results of measurements of factor VIII:C have been equally contradictory, with normal (Bensoussan et al., I975; Fuller et al., I979) and high levels (Pandolfi et al., I974; Gensini et al., I979) having been reported. The importance of the present study is that these changes appear to be reversible with metabolic control of the diabetes.

The cause of increased VIII:C activity during ketoacidosis is not known, but could be a non-specific response to acute stress (Brozovic, I977). Though increased concentrations of coagulation factors do not necessarily lead to an increased tendency to thrombosis Davis & McNicol (I978), the finding of a significantly shortened partial thromboplastin time during ketoacidosis suggests that there is increased activation of the intrinsic coagulation system, which might be expected to favour intravascular clotting. A slight, but significant, rise in the ratio of factor VIIIR:Ag/VIII:C was also observed during keto-acidosis. Denson has shown that a disproportionate rise in factor VIIIR:Ag relative to VIII:C can indicate intravascular coagulation (Denson, I977). However, since factor VIII:C is relatively more labile than VIIIR:Ag, the possibility that coagulant activity was lost during blood collection and storage cannot be discounted.

Since factor VIIIR:Ag is synthesized by vascular endothelial cells (Jaffe et al., I973), the high levels of this factor observed in the present study could reflect endothelial damage caused by metabolic factors during ketoacidosis. There is some evidence that antithrombin III

may also be synthesized by endothelial cells (Chan & Chan, 1979), so that the finding of reduced concentrations of antithrombin III during ketoacidosis is of interest. A fall in the concentration of antithrombin III appears to be an early and sensitive indicator of intravascular coagulation (Bick et al., 1977). In the present study, the observed fall in antithrombin III together with a modest rise in fibrin degradation products provides evidence that even in relatively uncomplicated cases of ketoacidosis, a degree of fibrin deposition occurs, and could contribute to the cerebral dysfunction often seen in this condition, as suggested by Timperley et al. (1974).

No relation was found between the magnitude of the individual haemostatic factor abnormalities and clinical and biochemical variables. Since levels of both haemostatic and metabolic factors can change rapidly, future studies with frequent blood sampling during recovery from ketoacidosis are required for a clearer understanding of the relationships between clinical, metabolic and haemostatic parameters.

Two of the three patients admitted with hyperosmolar comas died. Both deaths occurred in elderly patients, and illustrate the considerably higher mortality of this condition compared with ketoacidosis (Gordon & Kabadi, 1976). Both patients had laboratory, and in one case, pathological evidence of disseminated intravascular coagulation. The majority of patients with disseminated intravascular coagulation have reduced levels of fibrinogen and plasminogen, thrombocytopenia and diminished factor VIII:C and V with high levels of fibrin degradation products (Colman et al., 1972). These haematological findings do not clearly distinguish disseminated intravascular coagulation from massive thrombosis at a single site, such as was found in patient 14. Nevertheless, the persistant thrombocytopenia during this patient's final illness is suggestive of platelet consumption in sites additional to the occluded femoral artery.

As shown in the present study, coagulation factor levels are frequently raised during uncomplicated diabetic coma, which may obscure any changes induced by disseminated intravascular coagulation difficult to interpret. Thus, in previous reports of disseminated intravascular coagulation associated with diabetic coma, fibrinogen and factors V and VIII:C were high, low or normal (Table 20), in common with the two cases in the present series. Thrombocytopenia and raised titres of fibrin degradation products appear to be more consistent findings. In addition, a low plasminogen may point to the diagnosis.

The aetiology of disseminated intravascular coagulation during diabetic coma is likely to be multiple. Infection and shock are recognized precipitating factors (Colman et al., I972). Metabolic disturbances may play a part since disseminated intravascular coagulation is more readily induced in alloxan-treated compared to control rats (Antoniades et al., I973). In addition, Kwaan et al. (I972b) isolated a platelet aggregating-enhancing factor from a diabetic with diabetic coma who died with disseminated intravascular coagulation. The use of sensitive radioimmunoassays such as for fibrinopeptide A, beta-thromboglobulin and platelet factor 4 may provide information on the relative contributions of the coagulation system, platelets and endothelium to the pathogenesis of intravascular coagulation in diabetic coma.

In summary, this study has shown that during diabetic ketoacidosis, haemostatic changes are found which may reflect endothelial damage and also suggest a degree of fibrin deposition. In two fatal cases of the hyperosmolar syndrome, evidence of disseminated intravascular coagulation was found. In view of the high incidence of arterial thromboembolic complications associated with diabetic ketoacidosis and the hyperosmolar syndrome, controlled trials of platelet-inhibiting and/or anticoagulant drugs would seem to be justified in the management of these two conditions.

SECTION 2 EFFECT OF DIET AND GLICLAZIDE ON THE HAEMOSTATIC SYSTEM OF NON-INSULIN DEPENDENT DIABETICS

EFFECT OF DIET AND GLICLAZIDE ON THE HAEMOSTATIC SYSTEM OF NON-INSULIN DEPENDENT DIABETES

INTRODUCTION

Diabetic ketoacidosis represents a particularly severe stress to the body, so that it is perhaps not surprising that disturbances of the haemostatic system occur. Whether similar changes may be observed during the equilibration of hyperglycaemic, but not otherwise ill diabetic patients, was the objective of the present study.

The sulphonylurea gliclazide has recently been introduced into clinical practice in the United Kingdom. Apart from its hypoglycaemic properties, it has been claimed that administration of this drug to non-insulin dependent diabetics reduces platelet retention to glass bead columns (Rubinjoni et al., I978; Ponari et al., I979) and ADP and adrenaline-induced platelet aggregation (Ponari et al., I979).

However, these studies have not distinguished between a specific drug-induced effect on platelet function and the possible influence of improved glycaemic control brought about by the hypoglcaemic properties of this drug.

It was therefore decided to assess the effects of diet and gliclazide on a panel of haemostatic tests in newly-diagnosed patients with non-insulin dependent diabetes for a period of one year.

MATERIALS AND METHODS

Patients

Newly diagnosed non-insulin dependent diabetics were recruited for the study from the Diabetic Clinic at Leeds General Infirmary. There was no age limitation for the study, but patients already receiving other drug treatment, those who were expected to eventually require insulin therapy and grossly obese patients were excluded. A diagnosis of diabetes mellitus was made on the basis of a 50g two hour glucose tolerance test, using the criteria proposed by Keen et al. (I979). Patients with impaired glucose only, as defined by Keen et al. (I979), were also excluded.

The aims and the protocol of the study were explained

to each patient. If the patient agreed to participate in the study, blood was removed for haemostatic tests. The patient was then interviewed by the dietitian, who explained a conventional carbohydrate-restricted diet compatible with the patient's obesity and energy expenditure. In the initial phases of the study, the patient was encouraged to see the dietitian at two-weekly intervals, and thereafter every two months. After eight weeks dietary treatment, the patients underwent a second glucose tolerance test. If the two hr plasma glucose level exceeded 6mmol/l, in addition to the same diet, gliclazide was introduced, the dose being adjusted to give optimal control of the diabetes as judged by urine tests performed by the patient and 2hr post-prandial plasma glucose levels performed at two-monthly intervals at the diabetic clinic. Patients were asked to avoid all aspirin-containing compounds and were given paracetamol as a substitute.

Methods

Blood Sampling

Throughout the study, patients were reviewed at two-monthly intervals when non-fasting blood was removed using a I9 gauge "butterfly" from an antecubital vein using minimal stasis and a multiple syringe technique. As far as possible, the venesection was performed at the same time each day for each patient, and the order of syringes for the various tests remained constant. Platelet retention was performed immediately using fresh unanticoagulated blood. Blood for fibrinogen, heparin neutralizing activity, antithrombin III, factor VIII:C, factor VIIIR:Ag and euglobulin lysis times was taken into a 60ml syringe containing a solution of trisodium citrate in a volume of I : 9 volumes of blood, giving a final citrate concentration of 0.I29M. The citrated blood was mixed gently in the syringe and transferred to IOml plastic tubes and centrifuged within IO min at I,500 g. The plasma was stored until required at - 70°C, except for that required for measurement of the euglobulin clot lysis time, which was performed on fresh plasma. Blood for estimation of β-thromboglobulin was taken into the anticoagulant supplied with the assay kit and processed following the maker's instructions (The Radiochemical Centre, Amersham). Blood for plasma glucose was placed in a tube containing fluoride oxalate and serum for gliclazide levels was produced by incubating 5ml blood in a plastic tube at 37°C for one hr.

A plasma pool from 20 healthy male volunteers was obtained as detailed in Part II, Section 2.

Plasma glucose was measured using the glucose oxidase method.

Platelet Counts were measured using a Coulter Thrombocounter (Coulter Electronics Ltd., Harpenden, Herts.)

Factor VIII:C was measured by the one stage clotting assay previously described (Part II, Section 2) adapted for an automated coagulometer (Coag-a-Pet, General Diagnostics, Thame, Oxon.).

Euglobulin Clot Lysis Time was performed as previously described (Part II, Section 3), with the difference that clot lysis was determined visually using a transparent-sided waterbath.

Factor VIIIR:Ag was estimated by immunoelectrophoresis as previously described (Part V, Section I).

β-Thromboglobulin was measured using the radioimmunoassay provided by the Radiochemical Centre (Amersham).

Fibrinogen was measured as described previously (Part II, Section 2).

Heparin-neutralizing Activity was measured using the method described by Donati et al. (1972). In a water bath at 37°C , 0.05ml of heparin (concentrations of 0.1 - 4.0 IU /ml in 0.15M saline) was placed in a succession of tubes containing 0.25ml test plasma preincubated at 37°C for 1 min. 0.1ml bovine thrombin (Roche) at a concentration of 10 NIHu/ml was added and the clotting times of duplicate samples were recorded. When the clotting times were plotted against the concentration of heparin, a curve was obtained. The concentration of heparin producing a 20 s heparin clotting time was extrapolated from the curve, and the heparin-neutralizing activity was calculated from the formula :

$$\text{Heparin-neutralizing activity} = \frac{a \times b}{c}$$

where a = units of heparin giving a heparin clotting time of 20 s, b = volume of heparin and c = volume of the test plasma (Donati et al., 1972).

Antithrombin III was measured by radial immunodiffusion, as described in Part II, Section 3. Results were expressed as per cent of a normal plasma pool.

Per Cent Ideal Body Weight was calculated using Metropolitan Life Assurance tables (Documenta Geigy).

Platelet Retention was measured using a modification of the method of Salzman (1963). Seven ml venous blood was taken into a 5ml plastic syringe. Two ml was immediately expelled into a tube containing EDTA powder. The syringe was then swiftly attached to a polyvinyl catheter containing 1.5g of glass beads (0.5mm diameter) supplied by Servier Laboratories (Suresnes, France) and the assembly was placed on a constant rate pump (5ml in 46 sec), also supplied by Servier Laboratories. The 5ml of blood was forced through the glass bead column, and collected in a tube containing EDTA. A single batch of glass bead columns was used throughout the study. The drop in platelet count after passage through the column was expressed as a percentage of the platelet count before passage of the blood.

Serum Gliclazide was measured by Servier Laboratories UK (Horesdon, Surrey) using a gas-liquid chromatographic technique (Campbell et al., 1980). Samples were coded so that the technician who performed the measurements was unaware of the dose of gliclazide the patient was receiving.

STATISTICS

Comparisons were made with pre-treatment values using Wilcoxon's test for paired samples.

RESULTS

Fifteen patients were recruited into the study. One patient was withdrawn at the onset of gliclazide treatment because of dyspepsia (superficial gastritis shown by gastroscopy) which also recurred when another sulphonylurea, glipizide was introduced.

Clinical details of the I4 remaining patients are shown in Table 25. There were eight men and six women, with a mean age of 59.I years. Two patients presented with impaired vision and were found to have exudative retinopathy. Two had symptoms of peripheral neuropathy and the remainder had no clinical or electrocardiographic evidence of macro- or microvascular disease.

At diagnosis, the mean ideal body weight for height was II6.7 ± SEM 2.5% and after two months dietary treatment fell significantly to II0 ± 2.5% ($p < 0.0I$). However, at the end of the study, weight had increased significantly to II3 ± 3.4% compared to the mean at two months ($p < 0.0I$).

Three patients responded to dietary restriction alone and remained well-controlled throughout the study (mean postprandial plasma glucose at I2 months 4.3mmol/l). After two months of diet alone the remaining II diabetics were not satisfactorily controlled and gliclazide was introduced, daily doses ranging from 80 to 320mg/day.

Table 26 summarizes the laboratory findings in the II patients treated with diet and gliclazide. After two months of diet alone, significant reductions were observed in plasma glucose and factor VIII:C levels. Following the introduction of gliclazide, there was a further significant fall in plasma glucose. Platelet retention, factors VIII:C and VIIIR:Ag and plasma neutralizing activity were significantly reduced compared with pretreatment levels. These values remained relatively constant thereafter, though there was a tendency for platelet retention, factor VIII:C and factor VIIIR:Ag to increase towards the end of the study. Mean serum gliclazide concentrations remained constant throughout and the remaining haemostatic tests showed no significant changes. As shown in Fig. 30, when the per cent changes compared to pre-treatment values were plotted, platelet retention and factor VIIIR:Ag tended to parallel postprandial plasma glucose concentrations.

Because of the small number of patients adequately controlled on diet alone, statistical analysis of this group was not possible. Nonetheless, reductions in mean platelet retention, factors VIII:C and

VIIIR:Ag were observed as shown in Table 27 .

DISCUSSION

This study has shown that when gliclazide was added to the dietary treatment of newly-diagnosed non-insulin dependent diabetics who were not adequately controlled on diet alone, satisfactory glycaemic control was achieved. Reduction in blood glucose concentrations was associated with changes in tests of haemostatic function, namely a reduction in platelet retention, factor VIII:C, factor VIIIR:Ag and heparin-neutralizing activity. Smaller alterations in these tests occurred after the two months of diet alone, which with the exception of factor VIII:C, failed to reach statistical significance. Since the ideal weight was not achieved in all these patients, it is possible that an extension of the dietary period could have caused further falls in plasma glucose, with a corresponding improvement in haemostatic tests.

Towards the end of the I2 months, there was a tendency for the tests to return towards pretreatment values, whether the patients were treated by diet alone or by diet plus gliclazide. In the gliclazide treated group, mean serum drug levels remained constant, suggesting good compliance with therapy, but there was a significant weight gain and a slight rise in plasma glucose concentrations at I2 months which may imply a deterioration in dietary habits towards the end of the study.

At the time that this study was started, the measurement of glycosylated haemoglobin was not available in this laboratory. It may be that measurement of HbA_I is a more satisfactory method of assessing glycaemic control than postprandial plasma glucose estimations. However, unlike the situation in insulin-dependent diabetics where wide swings in plasma glucose may be expected, there is evidence that plasma glucose estimations generally correspond with HbA_I estimations in non-insulin dependent diabetics under stable conditions (Graf et al., I978).

Previous workers have shown that treatment of non-insulin dependent diabetics (previously inadequately controlled by diet alone, sulphonylureas or biguanides) with gliclazide resulted in reductions in platelet retention and platelet aggregation (Rubinjoni et al., I978;

Ponari et al., I979).However, in both studies, treatment with gliclazide (and probably also increased surveillance by the diabetic clinic) also produced an improvement in plasma glucose levels, and it is therefore uncertain whether changes in platelet function were due to the drug or to improved metabolic control. The results reported in the present study would appear to favour the latter hypothesis, since treatment with diet alone resulted in improvements in certain haemostatic tests, and falls in plasma glucose were parallelled by corresponding reductions in factor VIIIR:Ag and platelet retention.

The biological significance of such changes is uncertain. Evidence presented elsewhere in this Thesis suggests that haemostatic abnormalities tend to be most marked in diabetics with vascular complications. If an abnormal haemostatic mechanism plays a part in the pathogenesis of diabetic angiopathies, then a return of these tests towards normal might be expected to be beneficial. However, direct evidence that states of hypercoagulability or platelet hyper-reactivity cause thrombotic disease is not available. On the other hand, such abnormalities may be purely secondary to vascular damage. A hypothesis that vessel wall damage may be irreversible (eg microangiopathic changes) or reversible (eg during ketoacidosis or equilibration of poorly controlled diabetes), resulting in permanently abnormal or fluctuating levels and activities of haemostatic factors, has been postulated (Paton et al., I98I).

The relationship between platelet adhesion, platelet retention and factor VIII:vWf has been discussed in Part I, Section 2. A number of investigators have reported platelet retention to be raised in diabetic patients (Odegaard et al., I964; Hellem, I97I; Mayne et al., I970; Heath et al., I97I). In the majority of these studies, the increased retention was found to be related to the presence of microvascular complications. A relation between platelet retention and factor VIIIR:Ag has not been reported previously. Though platelet retention measures platelet-platelet as well as platelet-surface interactions, it seems probable that the reductions in platelet retention during treatment were at least partly caused by reductions in factor VIIIR:Ag. It has been previously observed that administration of glucose resulted in increased platelet adhesion to glass (Bridges et al., I965) in diabetics, and a high sucrose diet was reported to result in an increased platelet retention in a proportion of normal volunteers (Szanto & Yudkin, I969), which also suggests that changes in carbohydrate metabolism may influence the adhesive properties of platelets.

Factor VIIIR:Ag is known to be synthesized by vascular endothelial cells (Jaffe et al., I973b). Though a number of stimuli in vivo produce rises in this antigen (Brozovic, I977), to date stimulation of endothelial cells cultured in vitro has not resulted in increased synthesis of factor VIIIR:Ag (Tuddenham et al., I98I) so that the mechanism of control is unknown. The site of synthesis of factor VIII:C is not firmly established, but may be in the reticuloendothelial system (Bloom, I979). Rises of this factor may represent a non-specific response to tissue injury caused by the disordered carbohydrate and lipid metabolism in uncontrolled diabetes.

The ability of plasma to neutralize heparin is in part due to a factor released from platelets during the release reaction (platelet factor 4). Plasma levels of both heparin-neutralizing activity (Chmielewski & Farbizewski, I970) and platelet factor 4 (White & Marouf, I98I) have been reported in diabetic patients. However, a proportion of heparin-neutralizing activity is contributed by α-glycoproteins (Andersen & Godal, I977) which are also known to be increased in diabetic subjects (Jonsson & Wales, I976). Since β-thromboglobulin and platelet factor 4 are released simultaneously from platelets during the release reaction (Kaplan & Owen, I98I), the most likely explanation for the observed fall in heparin neutralizing activity would appear to be an alteration in α-glycoprotein components, since β-thromboglobulin levels did not alter throughout the study.

In conclusion, this study does not support the view that gliclazide per se exerts a beneficial effect on the hemostatic system of non-insulin dependent diabetics. However, control of plasma glucose levels with diet and gliclazide did lead to an improvement in some tests of coagulation and platelet function. Whether such changes are secondary to improvement in the health of the vascular endothelium, or whether such changes lead to a reduction in vascular complications must await the results of controlled clinical trials.

PART VI GENERAL DISCUSSION

GENERAL DISCUSSION

A brief resumé of the contents of this Thesis will be followed by a general discussion of the results in the context of diabetic vascular disease.

In Part I was discussed the possible pathogenetic factors of atherosclerotic and microvascular disease, and their relation to the high prevalence of vascular complications in diabetic patients. Evidence that the haemostatic system is involved in these two processes was reviewed. The implications of increased glycosylation of haemoglobin and other glycoproteins in diabetic subjects in relation to the assessment of diabetic control and possible pathogenetic mechanisms were discussed, followed by a review of platelet physiology and the coagulation and fibrinolytic systems, with particular emphasis on their possible roles in thrombogenesis and of attempts which have been made to detect a tendency to thrombosis.

In Part II, a cross-sectional study of platelet aggregation and coagulation and fibrinolytic factors was performed on a sample of patients attending a diabetic clinic. Compared with non-diabetic controls, no significant differences were observed in second-phase platelet aggregation, but significant increases in fibrinogen, factor II, V, VIII:C, α_2-macroglobulin and c_I -esterase inhibitor and reduced levels of factor XI and antithrombin III were found. In general, such changes were related to the presence of clinically-detectable large vessel disease, the prevalence of microangiopathy being low in this sample.

Part III was concerned with the development of a new method for measurement of platelet survival using an aspirin-labelling technique. With this non-radioisotopic method, platelet survival was found to be reduced in diabetic subjects compared with non-diabetic controls. ^{125}I-labelled fibrinogen binding to washed platelets and ADP-induced platelet aggregation in platelet-rich plasma (using a kinetic method of measurement) were found to be significantly increased in diabetic patients with severe retinopathy compared with diabetics with background and non-diabetic controls, illustrating that in vitro platelet behaviour is abnormal in diabetics with severe microvascular complications. A relationship between platelet aggregation and the concentration of glycosylated haemoglobin was also observed, suggesting that the state

of metabolic control may also influence platelet function. Evidence that the findings of reduced platelet survival and increased fibrinogen binding by platelets from diabetics may represent an alteration of the platelet membrane was discussed.

In Part IV, the possible role of the thromboxane-prostacyclin system in the modulation of platelet behaviour in diabetes was discussed. The effect of diabetic serum on the production of prostacyclin by cultured vascular endothelial cells was assessed. When the serum from diabetics with proliferative retinopathy was compared with serum from non-diabetic controls, an inhibitory effect on prostacyclin production was observed when added to the culture medium. This effect appeared to be related to the quality of metabolic control of the diabetes, but was not reproduced when a) glucose was added to the culture medium or b) when serum from well and poorly controlled diabeticswith no vascular complications were compared, suggesting that the presence of severe microangiopathy is required before an inhibitory factor is observed.

Part V examined two aspects of haemostatic abnormalities in relation to the metabolic control of diabetes. In contrast to the other work presented in this Thesis, these were prospective longitudinal studies. The first was designed to measure haemostatic factors during and after diabetic coma. During ketoacidosis and the hyperosmolar syndrome uncomplicated by clinically-evident thrombotic episodes, patients showed significant rises in factors VIIIR:Ag and VIII:C; and increases in fibrin degradation products together with a significant fall in antithrombin III which returned towards normal levels after re-equilibration of the diabetes. Evidence that these results represent signs of endothelial injury and intravascular fibrin deposition were presented. In two of the three deaths reported in this series, evidence of disseminated intravascular coagulation was found.

The second study was devised to observe the sequential changes in a panel of haemostatic tests during equilibration of newly-diagnosed non-insulin dependent diabetics with diet and the sulphonylurea gliclazide. Levels of factors VIIIR:Ag and VIII:C, and platelet retention and heparin-neutralizing activity were significantly reduced during treatment, the results tending to alter in parallel with the plasma glucose concentration.

In agreement with previously published studies, the results presented in this Thesis show that abnormalities of platelet behaviour

and altered levels of coagulation and fibrinolytic factors are observed in many diabetic subjects, and particularly in those patients with severe vascular complications. However, there is also evidence, presented in Parts III, IV and V of the Thesis, that the quality of glycaemic control is also capable of influencing some of these parameters. The relationship between metabolic control, alterations in haemostatic factors and the development of vascular complications is one of the major unsolved problems in diabetes.

It is possible that currently-available techniques are inadequate to completely resolve this problem. With the exception of the retina, assessment and quantification of micro and macrovascular disease by non-invasive means is difficult and imprecise. Patients (or controls) classified as being free of clinical vascular disease may have extensive atheroma affecting cerebral or coronary vessels. Doppler angiography and ultrasonic techniques have not yet been fully evaluated but may be of use in future studies. In the case of microvascular disease, a clinical search for evidence of microvascular disease cannot detect the early changes of basement membrane thickening. Thus subjects with abnormal haemostatic tests may be wrongly allocated to a group with "no vascular complications". In addition, tests of haemostatic function, particularly those related to platelet behaviour, are frequently poorly standardized between laboratories, making comparisons of the results of different research groups difficult. Moreover, the relation of these often highly unphysiological tests to in vivo thrombus formation is uncertain. Finally, diabetic patients comprise a heterogeneous group, particularly in regard to aetiology and pattern of inheritance (Irvine et al., 1977; Cudworth & Festenstein, 1978). Though the separation of diabetics into Type I (possessing islet cell antibodies, and typically insulin-dependent) and Type II (absence of islet cell antibodies, typically insulin-independent) has important implications for the aetiology of diabetes, both Type I and Type II develop vascular complications. However, since a proportion of diabetics escape vascular disease, even after many years of diabetes (Oakley et al., 1974; Paz-Guavera et al., 1975), it seems probable that genetic factors play a role in determining suceptibility to macro- and microangiopathy. That the importance of genetic susceptibility and control of glycaemia can be reconciled has recently been shown by Dornan et al. (1981) who found that the association of HLA type DR4 with poor diabetic control increases the risk of developing retinopathy.

For all these reasons, interpretation of cross-sectional studies which relate haemostatic tests to vascular complications is frequently difficult. Prospective, long-term population studies have been advocated as an alternative approach to determine whether abnormalities in haemostatic tests precede or follow the development of vascular complications; at least one such study is in progress (Fuller et al., I979). Yet any results which suggest that these tests predict macro or microvascular disease should be interpreted with caution, since sub-clinical vascular disease may be already present at the onset of the study.

The cause of the observed haemostatic abnormalities is also uncertain. Possible mechanisms for altered platelet behaviour are discussed in Parts III, IV and V of this Thesis. Evidence is presented that one factor may be an alteration in the platelet membrane, resulting in increased fibrinogen binding and shortened platelet survival. That a membrane abnormality is not restricted to platelets is suggested by the study of Wautier et al. (I98I) who showed that adhesion of erythrocytes from diabetic subjects to cultured endothelial cells was increased compared with controls. Moreover, this increased adhesiveness correlated with the extent and severity of vascular disease, but not with the degree of metabolic control as expressed by the concentration of HbA_I. It is also of interest that ^{51}Cr-labelled erythrocyte survival has been reported to be reduced in diabetic patients with severe microvascular disease (Brunning et al., I976). A hypothesis may be constructed to explain these findings : repeated cell-vessel wall interactions, particularly in areas where the vessel wall is irregular or denuded of endothelium produce damage or loss of part of the cell membrane, resulting in exposure of receptor sites (e.g. fibrinogen receptors in the case of platelets) and alterations of the surface properties of the cell membrane (e.g. increased adhesion of erythrocytes to endothelial cells) with accelerated removal of these cells from the circulation. Since the in vitro reactivity of newly-formed platelets appears to be higher than that of platlelets which have aged in the circulation (Karpatkin, I969; Ginsburg & Aster, I972), this may be another factor contributing to hyper-reactivity of platlets from diabetic subjects with vascular complications.

Metabolic influences may also influence platelet function, as suggested by the finding of a correlation between the sensitivity of ADP-induced platelet aggregation and HbA_I in Part III. Evidence from the animal studies cited in Part IV suggests that the balance between platelet

thromboxane synthesis and vessel wall production of prostacyclin is shifted towards thrombosis in rats made experimentally diabetic, this imbalance being corrected by treatment of the hyperglycaemia. This view receives some support from the results presented in Part IV which showed that both poor metabolic control of the diabetes and the presence of vascular complications needed to be present before a depressor effect of diabetic serum on endothelial cell prostacyclin production was observed.

An inter-relationship between hyperglycaemia, factor VIIIR:Ag and platelet retention was demonstrated in Part V, further illustrating the complexity of the factors affecting platelet behaviour. The results of Part V also show that certain alterations in tests of haemostatic function are at least partially reversible with improved diabetic control. Such tests may act as early markers of the degree of endothelial injury, and could provide an additional element to the assessment of diabetic control.

Whether these changes in haemostatic factors are involved in the pathogenesis of diabetic macro and microvascular disease is more speculative. As discussed in Part I, while there is reasonable experimental and pathological evidence for the involvement of platelet thrombi in the processes of atherogenesis and its thromboembolic complications, proof of a role in microvascular disease is scanty. One approach to assess the platelet contribution to microangiopathy could be to determine whether experimentally-induced diabetic animal models developed microangiopathy when rendered thrombocytopenic by anti-platelet serum. Alternatively, in a similar way to the study of the role of von Willebrand's disease in atheroma, it should be possible to establish an international register of diabetics with congenital bleeding disorders. As well as providing information on the genetic aspects of diabetes, it should be possible to discover whether these patients escape the vascular complications of diabetes.

In view of the difficulties of establishing a direct cause and effect relationship between haemostatic abnormalities and the pathogenesis of vascular disease in the diabetic, an indirect approach may be more fruitful. Certain drugs which have been shown to prolong a shortened platelet survival in patients with rheumatic or prosthetic heart valves such as dipyridamole (Harker & Slichter, I970) and sulphinpyrazone (Weily et al., I974) also appear to be effective in reducing the number of thromboembolic events (Sullivan et al., I97I). As platelet

survival is also reduced in diabetic patients (Part III), and can be prolonged by combined aspirin-dipyridamole therapy (Tindall et al., I98Ic), it may be justified to commence prospective trials of such drugs in the prevention of long-term vascular complications. Several studies are already in progress (Verstraete,I980). Anti-platelet prophylactic therapy, if proved successful, should not replace attempts to keep the control of glycaemia as close to normal as possible.

As opposed to the prevention of the long term complications of diabetes, the results presented in Part V argue strongly that in the acute metabolic dysequilibrium during diabetic coma, endothelial damage and fibrin deposition occur. In this situation, prospective studies of both anticoagulants and anti-platelet agents would appear to be justified to reduce the thromboembolic sequelae of this condition.

In conclusion, the results of this Thesis have shown that certain changes in haemostatic tests, and in particular tests of platelet behaviour, occur in diabetic patients. Some of these changes appear to be secondary to established vascular disease and others may be markers of endothelial injury, partially reversible by control of hyperglycaemia. The contribution of these abnormalities to the pathogenesis of micro- and macrovascular disease is still uncertain and further research in this area is required. At present, in the absence of reliable methods of identifying which patients will develop vascular disease, and for obtaining perfect metabolic control in all patients, clinical trials to assess the benefits of platelet inhibiting agents appear to be justified, and may indirectly provide evidence of the involvement of the haemostatic system in the pathogenesis of diabetic vascular disease.

SUMMARY

I. In a group of 34 diabetics, levels of fibrinogen, factors II, V, VII, VIII:C, α_2-macroglobulin and c_I-esterase inhibitor were significantly higher and concentrations of factor XI and antithrombin III were significantly lower than in 34 non-diabetic controls. α_2-macroglobulin levels were significantly higher in insulin dependent compared with insulin independent diabetics and c_I-esterase inhibitor concentrations were highest in those diabetics with vascular complications. The threshold concentrations of ADP, adrenaline and collagen required to produce second phase platelet aggregation was similar in diabetics and controls.

2. A non-radioisotopic method for measuring platelet survival employing aspirin as a label was refined. Using this technique, platelet survival times of I2 diabetics were found to be significantly shorter than those of I2 non-diabetic controls.

3. ^{I25}I-fibrinogen binding to platelets from 8 patients with severe retinopathy was significantly increased compared with 9 non-diabetic controls, and 8 diabetics with background retinopathy had fibrinogen binding values between the two groups. In the same study, using a kinetic analysis of the data, ADP-induced first phase platelet aggregation was significantly increased in the patients with severe retinopathy. In the diabetic patients, sensitivity of platelets to ADP was correlated with the concentration of haemoglobin A_I, but not with the degree of fibrinogen binding.

4. When the production of prostacyclin (PGI_2) by thrombin-stimulated human vascular endothelial cells was measured, cells cultured in the presence of 20% serum from diabetics with proliferative retinopathy generated significantly less PGI_2-like activity and 6-keto-$PGF_{I\alpha}$ compared with cells cultured with control serum. Diminished 6-keto-$PGF_{I\alpha}$ production was correlated with the concentration of haemoglobin A_I. However, the same effect was not observed when the culture medium was supplemented with a) different concentrations of glucose and b) when sera from well controllled diabetics were compared with serum from poorly controlled diabetics without vascular complications.

5. A study of haemostatic factors in I5 patients admitted with diabetic coma (II with ketoacidosis and 3 with the hyperosmolar syndrome) was performed. Compared with values after recovery, during ketoacidosis, significant increases in factors VIII:C and VIIIR:Ag, fibrin degradation products and reduced concentrations of antithrombin III were observed. Out of 3 deaths in the series, 2 had evidence of disseminated intravascular coagulation.

6. A panel of haemostatic tests were performed in I4 newly-diagnosed non-insulin dependent diabetics. After 2 months of treatment with diet alone, II were given the sulphonylurea gliclazide, the three others remaining on dietary treatment. Compared with pre-treatment values, significant reductions in platelet retention, factors VIII:C and VIIIR:Ag and plasma heparin-neutralizing activity accompanied a fall in plasma glucose due to diet or to diet plus gliclazide.

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ABBREVIATIONS

ADP	adenosine diphosphate
BP	blood pressure
BPR	basic platelet reaction
cm	centimetre
CVD	cerebrovascular disease
cyclic AMP	3' 5'-cyclic adenosine monophosphate
dl	decilitre
ECG	electrocardiograph
EDTA	ethylenediamine tetraacetic acid
ELT	euglobulin clot lysis time
F	(coagulation) factor
Fig	Figure
g	gram
g	gravitation field
h	hour
Hb	haemoglobin
HEPES	hydroxy-2-ethyl-piperazinyl-I-2-ethane-sulphonic acid
IHD	ischaemic heart disease
kg	kilogram
l	litre
M	molar
MDA	malondialdehyde
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimole
mmHg	millimetres of mercury
mOsmol	milliosmole
N	normal
ng	nanogram
NIHu	National Institute of Health unit
nm	nanometre
nM	nanomolar
nmol	nanomole
OD	optical density
PAS	periodic acid Schiff

PF4	platelet factor 4
PG	prostaglandin
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PT	prothrombin time
PTT	partial thromboplastin time
PVD	peripheral vascular disease
rpm	revolutions per minute
s	second
SD	standard deviation
SEM	standard error of the mean
TEP	I,I,3,3-tetraethoxypropane
Tris	tris(hydroxymethyl)aminomethane
u	unit
U	international unit
uM	micromolar
umol	micromole
um	micron
ug	microgram
VIII:C	(factor) VIII coagulant activity
VIIIR:Ag	(factor) VIII-related antigen
VIII:vWf	(factor) VIII-von Willebrand factor
w/w	weight for weight

CO-WORKERS

The work presented in this Thesis was undertaken independently and personally with the following exceptions.

Coagulation and fibrinolytic assays were performed by the technical staffs of the Departments of Aberdeen and Leeds.

^{125}I-fibrinogen binding assays were partly performed by myself and partly by Dr H Lee and Mme A Thomaides, Hôpital St Louis, Paris.

Cultures of vascular endothelial cells were produced by Dr R Guillot, Department of Embryology, UER Biomédicale, rue des Saints-Pères, Paris.

Statistics were calculated by me, though I received advice from Dr S Gore, Department of Statistics, Aberdeen University.

PLACE OF WORK AND APPOINTMENTS

The work for this Thesis was started during my tenure as a Research Fellow in the Department of Medicine, Aberdeen University between October 1975 and August 1977. It was continued during my appointment as Tutor and subsequently Lecturer in the Department of Medicine, University of Leeds from September 1977, and was completed during sabbatical leave from October 1979 until September 1981 with the Department of Endocrinology and Metabolism and the Laboratories of Haemostasis and Experimental Thrombosis, Hôpital St Louis, Paris.

PUBLICATIONS RELATED TO ORIGINAL WORK PRESENTED IN THIS THESIS

PATON RC (I979)
Platelet survival in diabetes mellitus using an aspirin-labelling technique. Thrombosis Research I5: 793-802.

PATON RC (I98I)
Haemostatic changes in diabetic coma. Diabetologia 2I: I72-I77.

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Effects of diet and gliclazide on the haemostatic system of non-insulin-dependent diabetics. British Medical Journal 2: I0I8-I020.

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Fibrinogen binding and ADP-induced aggregation in platelets from diabetic subjects. Thrombosis Research.

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PLATELET SURVIVAL IN DIABETES MELLITUS USING AN ASPIRIN-LABELLING TECHNIQUE

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ABSTRACT

Using an aspirin-labelling technique, platelet survival was measured in twelve diabetics and twelve non-diabetic controls. Platelet survival times were significantly shorter in the diabetics compared to the controls ($p<0.01$). The shortening did not appear to correlate with the presence of diabetic vascular complications.

INTRODUCTION

Diabetes mellitus is characterised by a susceptibility to atherosclerotic and microvascular disease. Platelets may be important in the production of these complications (1). When tested *in vitro* diabetic platelets show many abnormalities including enhanced retention to glass surfaces (2,3), increased sensitivity to aggregating agents such as adenosine diphosphate, adrenaline and collagen (4-6), and increased pro-aggregatory prostaglandin production (7).

Fewer studies have examined the behaviour of diabetic platelets *in vivo*. Using ^{51}Cr-sodium chromate, Abrahamsen (8) found platelet survival to be reduced in juvenile-onset diabetics with microvascular complications and Ferguson et al (9), using ^{75}Se-selenomethionine, showed juvenile-onset diabetics without obvious complications had shorter platelet survival times than controls.

Wider application of this technique has been hindered by the fact that radioisotopic labels are expensive and special equipment and expertise is required. Direct labels (e.g. ^{51}chromium) involve the handling and possible damage of platelets ex-vivo, whereas in vivo labels (e.g. ^{75}selenium) usually undergo re-utilisation, making interpretation of results difficult (10).

An alternative non-radioisotopic method was suggested by Stuart et al (11) which used aspirin as the platelet label. The results appear to correlate with those obtained using ^{51}chromium (11,12). A modification of this technique, evolved in the University Department of Medicine, University of Leeds, was used to compare platelet survival in diabetic and control subjects.

Subjects

Twelve non-diabetic controls (7 men; 5 women) and 12 diabetics (5 women; 7 men) were studied. The controls were healthy laboratory and medical staff with a mean age of 30.8 years (range 23-49 years). The diabetics, with two exceptions, were fully ambulant and had a mean age of 39.4 (range 16-65). Clinical details of the diabetics are shown in table 1.

TABLE 1

Subject	Sex	Age (yrs)	Duration Diabetes (yrs)	Treatment	Complications
EW	F	40	11	Insulin	Nephrotic syndrome, neuropathy
CW	F	34	11	Insulin	Retinopathy, proteinuria, neuropathy
MC	F	45	1	Metformin	None
MF	F	37	12	Insulin	None
AW	M	50	8	Tolazamide	Nephrotic syndrome, gangrenous toe
AG	M	47	2	Metformin	BP $^{174}/_{94}$
AMcI	F	20	0.5	Insulin	None
JM	M	41	9	Insulin	Neuropathy
RW	M	40	16	Insulin	Neuropathy, ischaemic heart disease
JM	M	65	0.5	Insulin	Neuropathic ulcer, ischaemic heart disease
CM	M	49	6	Chlorpropamide	None
MD	M	16	11	Insulin	None

Clinical Details of Diabetic Subjects

None of the subjects were taking any medications known to influence platelet behaviour. None of the female subjects were menstruating at the time of study. On the days of blood sampling, diabetics had their normal antidiabetic therapy.

METHODS

a) Estimation of platelet MDA production

Non-fasting blood was collected into plastic tubes containing $^1/_{10}$ volume 0.77M EDTA. The tubes were centrifuged at 250g for 10 minutes to yield platelet-rich plasma (PRP). Platelet count was measured using the method of

Brecher & Cronkite (1950) (13). 1 ml aliquots of PRP were centrifuged at 500 g for 12½ minutes and the resulting platelet-poor plasma was poured off, and the inside of the tube carefully dried. The platelet buttons were re-suspended in 1 ml phosphate-saline buffer, pH 7.4, using magnetic stirrers and polythene-coated magnets. 0.1 ml of a 20 mM solution of n-ethyl maleimide was added to the platelet suspensions and the tubes were incubated at 37°C for 30 minutes which results in the formation of malondialdehyde (MDA). The reaction was stopped and protein precipitated by the addition of 0.2 ml 20% trichloracetic acid dissolved in 0.6 M hydochloric acid. The tubes were re-centrifuged at 1000 g for 15 minutes and 1 ml of supernatant was placed in boiling tubes with 0.2 ml of 1% sodium thiobarbiturate. The tubes were then boiled for 15 minutes, cooled, and the optical density of the resulting pink colour was measured at a wavelength of 532 nm, using a Pye Unicam SP 1800. Results were arbitrarily expressed as optical density per 10^9 platelets.

b) Estimation of platelet survival

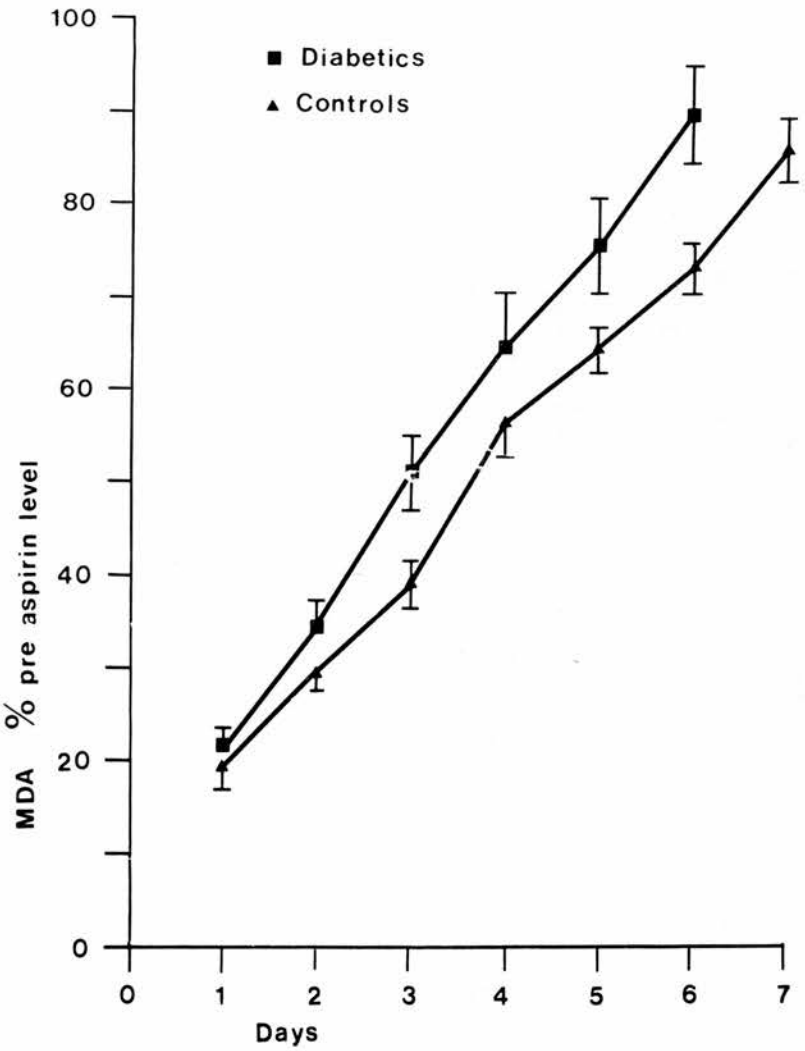
MDA production was estimated in platelets stimulated with n-ethyl maleimide as described, in subjects who had taken no drugs known to affect platelet function in the preceding two weeks. Preliminary studies showed day-to-day variation in MDA production was small (coefficient of variation 4%). The subjects were then given 600 mg soluble aspirin and MDA production was measured at daily intervals until values exceeded 80% of pre-aspirin levels. Results were expressed as a percentage of the pre-aspirin level and plotted against time. For each subject a straight line was computed using linear regression analysis and platelet survival was expressed as time in days where the line reached 100% of the pre-aspirin level. Platelet survival times were compared using Wilcoxon's rank sign test.

RESULTS

Fig. 1 shows the mean values of MDA production expressed as a percentage of the pre-aspirin level in diabetics and controls.

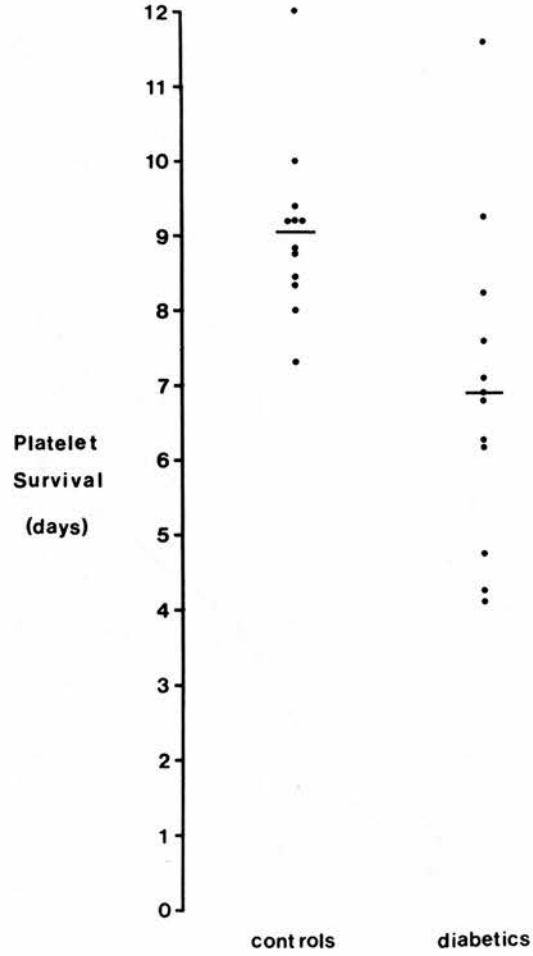
Fig. 2 shows the individual platelet survival times.

FIG 1



MDA production in controls and in diabetics after aspirin intake (mean ± SEM)

FIG 2



Platelet Survival in Diabetics and in Normal Control Subjects

The mean platelet survival time of the diabetics was 6.92 days, which was significantly shorter ($p<0.01$) than the mean of 9.05 days found in the controls. Platelet survival did not appear to be related to the presence of vascular complications, and was not correlated with age or sex of the

patient or duration of diabetes.

DISCUSSION

The aspirin labelling method is a cheap and simple alternative to radiolabelling techniques. The major advantage is that labelling takes place in vivo, obviating possible platelet damage during handling. An additional advantage is that since there is no radiation hazard, studies may be repeated in the same individual and the technique might also be of use during pregnancy. Unlike radioisotopic methods the technique cannot be used when the subject is taking a drug which inhbits MDA production, e.g. non-steroidal anti-inflammatory drugs, and since it actually measures platelet renewal rather than survival, platelet production must equal platelet destruction as reflected by a constant platelet count throughout the period of study.

Shortened platelet survival has been found in a variety of conditions associated with thromboembolism including rheumatic heart disease (14), prosthetic heart valves (15-17), familial hypercholesterolaemia (12), and coronary artery disease (18,19). In patients with prosthetic heart valves and in rheumatic mitral valve disease, the frequency of thromboembolic events has been related to shortened platelet survival (14,17). The present study confirms earlier work (8,9) that platelet survival is shortened in some diabetics. The cause of shortened survival times is unclear. Platelets from diabetics appear to be unduly sensitive to aggregating agents (4-6), and therefore may be more prone to participate in thrombus formation. In addition the vascular endothelium in diabetic patients may be abnormally thrombogenic. Atheroma is more widespread in diabetics (20) and could enhance a tendency to thrombus formation. In addition it has recently been shown that production of the anti-aggregatory prostaglandin I_2 by the vascular endothelium of diabetic patients is

impaired (21).

One patient with a greatly reduced platelet survival of 4.25 days, was newly-diagnosed and undergoing stabilisation at the time of the study. Preston et al (22) have shown that β-thromboglobulin levels fall when the blood sugar is lowered in newly diagnosed diabetes and it is possible that poor diabetic control may be associated with shortened platelet survival.

It has been suggested that shortened platelet survival may identify proneness to thrombosis in groups of patients, and in whom antithrombotic therapy would be particularly indicated (23). Drugs such as dipyridamole (16) and sulphinpyrazone (17) have been shown to prolong shortened platelet survival in patients with prosthetic heart valves and clinical studies are in progress to evaluate their effectiveness in coronary artery and cerebrovascular disease. Clinical trials of such drugs to assess their ability to prevent the vascular complications in diabetes may be indicated.

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Originals

Haemostatic Changes in Diabetic Coma

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Summary. Diabetic coma is frequently associated with thromboembolic complications. A prospective study was undertaken of the haemostatic changes occurring in 15 patients (12 with ketoacidosis, three with the hyperosmolar syndrome) during diabetic coma. When compared with the results after stabilization of the diabetes, ketoacidosis was associated with significantly higher levels of factor VIII coagulant activity, factor VIII-related antigen and fibrin degradation products, a shorter partial thromboplastin time and reduced concentrations of antithrombin III. These results suggest that in uncomplicated ketoacidosis, haematological changes occur which may reflect vascular endothelial damage and intravascular fibrin deposition. Out of three deaths, two patients (both with the hyperosmolar syndrome) had evidence of disseminated intravascular coagulation. To reduce further the mortality and morbidity from diabetic coma, controlled clinical trials of anticoagulant and antiplatelet drugs may be indicated.

Key words: Diabetic coma, factor VIII, disseminated intravascular coagulation.

Many abnormalities of haemostatic function have been described in diabetes mellitus. These include increased platelet retention and aggregation [1], increased levels of coagulation factors [2] and diminished fibrinolytic activity [3]. Whether these abnormalities are related to the metabolic disturbances of diabetes is unclear.

Whatever the relationship between metabolic control and haemostatic dysfunction, it is increasingly realized that thromboembolic disease is a frequent complication of diabetic coma. Arterial thrombosis may be responsible for up to one-third of deaths in patients with diabetic ketoacidosis [4], and is a frequent occurrence in non-ketotic hyperosmolar coma [5]. In addition, Timperley et al. have shown that localized deposition of fibrin may occur in fatal cases of ketoacidosis, particularly in the cerebral capillaries [6]. Over the past decade, a number of authors have described the association of disseminated intravascular coagulation and diabetic coma [6–8]. However, such case reports have been sporadic, and laboratory studies have generally been undertaken only after clinical signs were apparent. A prospective study was therefore designed to determine the nature of haemostatic changes in unselected cases of diabetic ketoacidosis and the non-ketotic hyperosmolar syndrome.

Subjects and Methods

Patients

Fifteen diabetic patients, admitted to the Aberdeen Teaching Hospitals with ketoacidosis or the hyperosmolar syndrome over a period of 12 months, were included in the study. Diabetic ketoacidosis was arbitrarily defined as a plasma glucose >14 mmol/l and a serum bicarbonate of 11 mmol/l or less in association with ketonuria. The non-ketotic hyperosmolar syndrome was defined as a plasma glucose >30 mmol/l with a serum osmolarity of >350 mOsmol/l [9], serum bicarbonate >20 mmol/l and with no more than a trace of ketonuria.

Where possible, a second, "convalescent" sample was obtained from the patients at a subsequent outpatient visit when the diabetic state was stable (non-fasting plasma glucose <10 mmol/l and absence of ketonuria).

Methods

Venous blood was removed from the patients as soon as possible after arrival at hospital (mean 2.2 h, range 0.1–5 h) for biochemical and haematological studies. Blood for assays of the coagulation

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Table 1. Clinical details of patients with diabetic coma

Patient no.	Sex	Age (years)	Duration of diabetes (years)	Previous diabetic treatment	Possible precipitating factors	Additional drugs	Depth of coma	Outcome
1	M	18	4	insulin	tonsillitis	erythromycin	3	recovery
2	F	52	new	none	breast abcess	amoxycillin, bendrofluazide K^+	1	recovery
3	F	65	46	insulin	unknown	–	4	death
4	F	21	10	insulin	unstable diabetes	–	3	recovery
5	M	17	new	none	unknown	–	4	recovery
6	F	15	6	insulin	unstable diabetes	–	2	recovery
7	F	13	1	insulin	dental abcess	–	2	recovery
8	M	33	13	insulin	ischiorectal abcess	–	2	recovery
9	F	16	1	insulin	unstable diabetes	–	2	recovery
10	M	17	new	insulin	stopped insulin	–	3	recovery
11	F	33	new	none	unknown	–	2	recovery
12	M	42	16	insulin	unknown	–	3	recovery
13	M	65	new	none	unknown	–	3	recovery
14	M	73	new	none	unknown	frusemide, digoxin, inositol nicotinate	3	death
15	F	81	17	chlorpropamide	stopped sulphonylurea	–	3	death

Table 2. Biochemical data of patients with diabetic coma

Patient no.	Plasma glucose (mmol/l)	Serum urea (mmol/l)	Serum sodium (mmol/l)	Serum potassium (mmol/l)	Serum bicarbonate (mmol/l)	Serum osmolarity (mOsmol/l)	Urinary ketones
1	32.4	9.4	128	5.4	5	309	+++
2	21.4	8.0	151	2.4	8	336	+++
3	54.9	14.7	132	7.2	5	348	+++
4	14.2	5.5	153	6.5	5	339	+++
5	78.5	38.0	146	4.4	11	417	+++
6	39.3	6.8	136	5.5	5	329	+++
7	25.0	4.0	133	3.7	5	302	+++
8	34.6	8.3	134	6.6	5	324	+++
9	17.1	8.2	142	4.6	11	318	++
10	20.9	5.6	136	3.5	5	305	+++
11	42.9	7.0	138	2.5	5	331	+++
12	69.0	39.0	143	6.5	11	407	++
13	31.1	18.2	161	4.1	24	380	–
14	56.1	35.2	145	5.7	22	393	trace
15	48.4	28.1	163	4.0	24	410	–

system was immediately placed on ice and centrifuged at 1500 × *g* and 4 °C within 30 min of venepuncture. Prothrombin and partial thromboplastin times were measured on fresh samples and the remaining plasma stored at −70 °C until required.

The whole blood platelet count was measured microscopically [10]. Partial thromboplastin time was measured by the method of Langdell et al. [11] and the prothrombin time by the method of Quick [12]. Factor V and factor VIII coagulant activity (VIII: C) were assayed by one-stage techniques based on the correction of the prolonged clotting times of specific factor deficient plasmas [13, 14]. Factor VIII-related antigen (VIII R: Ag) was measured by immuno-electrophoresis using antibody to factor VIII R: Ag produced by Behringwerke (Marburg, FRG) [15]. A normal pool of plasma obtained from 20 healthy male medical students was used as a control. Plasma antithrombin III was measured by a radial diffusion technique, using plates and standards supplied by Behringwerke. Plasma fibrinogen was measured by a modification [16] of the method of Ratnoff and Menzie, and plasma plasminogen by the method of Alkjaersig et al. [17]. Fibrin degradation products were measured using the kit supplied by Wellcome Reagents (Beckenham, Kent, UK) Packed cell volume was estimated using a microhaematocrit centrifuge (Hawksley & Sons, Lancing, Sussex, UK). Since dehydration occurs during diabetic coma, concentrations of haemostatic factors were adjusted for changes in haematocrit [18]: Corrected concentration = measured concentration $\times \frac{H_1 (100 - H_2)}{H_2 (100 - H_1)}$

Where: H_1 = haematocrit after coma
H_2 = haematocrit during coma

This formula is based on the assumption that the red cell mass remains relatively constant during diabetic coma, and that changes in haematocrit reflect changes in plasma volume. Results are pre-

Table 3. Mean ± SEM values of haemostatic factors of 11 diabetics during ketoacidosis, ketoacidosis with the values corrected for dehydration and during convalescence

Test	Ketoacidosis (measured values)	Ketoacidosis (corrected values)	Convalescence	Normal controls[a]
Haematocrit (%)	45.6±1.8[d]		40.7±1.5	
Prothrombin time (ratio)	1.00±0.03		0.96±0.04	
Partial thromboplastin time (ratio)	0.90±0.04[c]		0.99±0.03	
Platelet count (x 10^9/l)	406±43[c]	331±31	299±17	
Factor V (%)	110±10	90±9	118±15	
Factor VIII: C (%)	350±36[b]	278±33[d]	189±23	
Factor VIIIR: Ag (%)	535±156[b]	511±226[b]	125±10	
Fibrinogen (g/l)	6.35±0.63	5.63±1.02	5.19±0.32	3.92±1.35
Plasminogen (casein U/ml)	4.19±0.16	3.51±0.29[d]	4.09±0.17	3.56±0.07
Antithrombin III (mg/dl)	32.2±1.4[b]	26.4±1.0[b]	36.3±0.3	46.5±1.0
Fibrin degradation products (μg/ml)	17.7±4.5[d]	15.4±4.4	8.7±0.9	<5

[a] Mean ± SEM values for 34 healthy non-diabetic subjects.
[b] $p < 0.01$, [c] $p < 0.02$, [d] $p < 0.05$ compared with convalescent values

Table 4. Haemostatic tests of the fatal case of ketoacidosis (patient 3) and the three diabetics with the hyperosmolar syndrome (patients 13, 14, 15)

Test	Patient 3	Patient 13	Patient 14	Patient 15	Normal control[a]
Haematocrit (%)	40	40	49	39	
Prothrombin time (ratio)	1.01	1.01	1.41	0.88	
Partial thromboplastin time (ratio)	1.33	0.60	0.98	0.83	
Platelet count (x 10^9/l)	540	311	82	184	
Factor V (%)	104	102	42	104	
Factor VIII: C (%)	240	570	184	760	
Factor VIIR: Ag (%)	248	280	352	1200	
Fibrinogen (g/l)	5.20	10.15	10.32	4.25	3.92±1.35
Plasminogen (casein U/ml)	3.31	4.34	2.75	1.76	3.56±0.07
Antithrombin III (mg/dl)	27.2	27.2	26.0	27.7	46.5±1.0
Fibrin degradation products (μg/ml)	20	10	160	80	<5

[a] Mean ± SEM values for 34 healthy non-diabetic subjects

sented both as measured and corrected concentrations. Plasma glucose was measured by the glucose oxidase method and serum urea and electrolytes by autoanalyzer. Urinary ketones were measured by Ketostix (Ames). Serum osmolarity was calculated using the formula of Gordon and Kabadi [9], and depth of coma on admission was assessed clinically, using a score of 1–4 (1 = normal, 2 = drowsy, but responding to verbal commands, 3 = responding only to painful stimuli, 4 = unrousable).

Statistics

Comparison between results during and after diabetic coma was made using the Wilcoxon rank sign test for paired samples.

Results

Patients

Fifteen diabetics were admitted to the study. Clinical details are shown in Table 1 and biochemical data in Table 2. Findings in the 12 patients with ketoacidosis are discussed separately from the three patients with the hyperosmolar syndrome.

Patients with Ketoacidosis

Patient 3 died 3 h after admission following a cardiac arrest. The results of the haemostatic tests of the surviving 11 patients are summarized in Table 3. The normal values for fibrinogen, plasminogen, antithrombin III and fibrin degradation products shown in Tables 3 and 4 were obtained from 34 healthy non-diabetic controls (22 men, 12 women) with a mean age of 39.1 years (range 26–65 years). Compared with convalescent values, platelet count, factor VIII: C, VIII R: Ag and fibrin degradation products were significantly increased. Antithrombin III concentrations were significantly lower and there was a significant shortening of the partial thromboplastin time during ketoacidosis.

When the results were corrected for the effect of dehydration, factor VIII: C and VIII R: Ag were still significantly higher and antithrombin III concentrations lower, during ketoacidosis. The differences in platelet count and fibrin degradation products were no longer significant, though seven out of the 11 patients had corrected concentrations of fibrin degradation products greater than 10 μg/ml compared with only one out of 11 convalescent samples. In addition, corrected mean plasminogen concentrations were significantly lower during ketoacidosis. The mean (± SEM) factor VIIIR: Ag/VIII: C ratio was 1.44 ± 0.27 during ketoacidosis, which was significantly higher than the convalescent value of 0.73 ± 0.08 ($p < 0.01$). There was no correlation between the levels of the various haemostatic factors and the depth of coma, the presence of overt infection, plasma glucose, serum bicarbonate and the serum osmolarity. Neither the two patients with severe hypokalaemia (patients 2 and 11) nor the two with a blood urea greater than 30 mmol/l (patients 5 and 12) had haemostatic values strikingly different from the remaining ketoacidotic patients.

Patients with the Hyperosmolar Syndrome

Out of the three diabetics with the hyperosmolar syndrome, two died (patients 14 and 15). The haemostatic results on admission are shown in Table 4, together with the fatal case with ketoacidosis (patient 3). Results are reported as uncorrected values. Patients 3 and 13 showed results similar to the surviving patients with ketoacidosis shown in Table 3. In contrast, the two fatal hyperosmolar cases (patients 14 and 15) had thrombocytopenia, low concentrations of plasminogen and raised levels of fibrin degradation products.

Patient 14 had a past history of ischaemic heart disease and intermittent claudication. On admission, he was found to have impalpable pulses from the left femoral downwards. The hyperosmolar state was rapidly corrected with fluid and insulin therapy, but perfusion of the left leg did not improve, below-knee amputation being required six days later, followed by death after a further eight days. The severe thrombocytopenia present on admission persisted throughout his final illness. Post-mortem was not performed.

Patient 15 was admitted in hyperosmolar coma. On examination she had minimal abdominal tenderness and absent bowel sounds. Death occurred 12 h after admission. At autopsy she was found to have 80 cm of infarcted small intestine and when sections were stained with Martius yellow, Crystal scarlet and soluble blue, fibrin was observed in capillaries, venules and arterioles of lungs, mesentry, renal glomeruli and cerebral cortex.

Discussion

This study shows that characteristic changes occur in the haemostatic system during diabetic ketoacidosis, the most striking abnormality being a rise in factor VIII: C and VIII R: Ag. Increased levels of the factor VIII complex are found in a variety of inflammatory, thromboembolic and neoplastic conditions [19]. On the other hand, high factor VIII: C and particularly VIII R: Ag concentrations have frequently been observed in stable diabetics [20, 21]. Recent evidence suggests that factor VIII Ristocetin cofactor activity (which tends to parallel the VIII R: Ag level) can be reduced by strict metabolic control of the diabetes [22]. The present study lends additional support to the view that poor diabetic control produces raised factor VIII levels. Since factor VIII R: Ag is synthetized by vascular endothelial cells [23], the high concentrations of this antigen found in diabetics with ketoacidosis may indicate the degree of insult sustained by the endothelium. Exposure of the vascular subendothelium is the first stage in the initiation of thrombus formation [24], so that endothelial cell damage with resulting de-endothelialization could be a major contributing factor to the high incidence of arterial thrombosis in fatal cases of diabetic ketoacidosis [4].

The cause of raised factor VIII: C activity during ketoacidosis is not known, but could be a non-specific response to acute stress [19]. Though raised levels of individual coagulation factors do not necessarily lead to an increased tendency to thrombosis [25], the finding of a significantly shortened partial thromboplastin time during ketoacidosis suggests that there is an increased activity of the intrinsic coagulation system, which might be expected to favour intravascular clotting. A slight, but significant, rise in the ratio of factor VIII R: Ag/VIII: C was observed during ketoacidosis. Denson has shown that a proportionate decrease in factor VIII: C in relation to VIII R: Ag can indicate intravascular coagulation [26]. However, since factor VIII: C activity is relatively labile, the possibility that coagulant activity was lost during blood collection or storage of the plasma cannot be excluded.

A fall in the concentration of antithrombin III appears to be an early and sensitive indicator of intravascular coagulation [27]. In the present study, a significant fall in antithrombin III together with a modest rise in fibrin degradation products provides evidence that even in relatively uncomplicated cases of ketoacidosis, a degree of fibrin generation occurs, and could contribute to the cerebral dysfunction often seen in this condition [6]. No correlation was found between the magnitude of the individual

haemostatic factor abnormalities and clinical and biochemical variables such as presence of overt infection, depth of coma, extent of hyperglycaemia, osmolarity or uraemia. Since levels of both haemostatic and metabolic factors can change rapidly, future studies with more frequent blood sampling during recovery from ketoacidosis are required for clearer understanding of the relationships between clinical, metabolic and haemostatic parameters.

Two of the three patients with the hyperosmolar syndrome died during their admission to hospital. Both deaths occurred in elderly patients and illustrated the considerably higher mortality rate due to the hyperosmolar syndrome compared with that from ketoacidosis [9]. Both patients had laboratory, and in one case, pathological evidence of disseminated intravascular coagulation. The majority of patients with disseminated intravascular coagulation have reduced levels of fibrinogen and plasminogen, thrombocytopenia and diminished factor V and VIII: C with high levels of fibrin degradation products [27, 28]. These haematological findings do not clearly distinguish disseminated intravascular coagulation from massive thrombosis at a single site, as was found in patient 14. Nevertheless, the persistent thrombocytopenia found in this patient is suggestive of platelet consumption in sites additional to the occluded femoral artery.

As shown in the present study, coagulation factor levels are frequently raised during uncomplicated diabetic coma, making any changes induced by disseminated intravascular coagulation difficult to interpret. Thus in previous reports of disseminated intravascular coagulation associated with diabetic coma, fibrinogen and factors V and VIII: C were high, low or normal [6–8], in agreement with the two cases in the present series. Thrombocytopaenia and raised fibrin degradation products appear to be more consistent findings. In addition, a low plasma plasminogen concentration may point to the diagnosis.

The aetiology of disseminated intravascular coagulation during diabetic coma is likely to be multiple. Infection [27, 28] and hypovolaemia [28] are known precipitating factors. Metabolic abnormalities may also play a part since disseminated intravascular coagulation is more readily induced in alloxan-treated compared with control rats [29]. The use of sensitive radioimmunoassays such as those for fibrinopeptide A, beta-thromboglobulin and platelet factor 4 may provide information on the relative contributions of the coagulation system, platelets and endothelium to the pathogenesis of intravascular coagulation during diabetic coma.

In summary, this study shows that during diabetic ketoacidosis, haemostatic changes are found which may reflect endothelial damage and also suggest intravascular fibrin deposition. In two fatal cases of the hyperosmolar syndrome, evidence of disseminated intravascular coagulation was found. In view of the high incidence of thromboembolic complications associated with diabetic ketoacidosis and the hyperosmolar syndrome, and the disturbances of the haemostatic system reported in this paper, controlled clinical trials of anticoagulant and antiplatelet drugs would appear to be justified in the management of these two conditions.

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Effects of diet and gliclazide on the haemostatic system of non-insulin-dependent diabetics

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Abstract

The effect of the sulphonylurea gliclazide on tests of haemostatic function was studied in 14 newly diagnosed non-insulin-dependent diabetics. After two months' treatment with diet alone 11 of the 14 were given gliclazide; the three others remained on dietary treatment. Compared with pretreatment values, significant reductions in platelet retention, factor VIII-related antigen, factor VIII coagulant activity, and plasma heparin neutralising activity accompanied a fall in the plasma glucose concentration due to either diet alone or diet and gliclazide.

The beneficial effects of gliclazide on platelet abnormalities seem likely to be due to its hypoglycaemic action rather than to any direct effect on haemostatic function.

Introduction

Abnormalities of platelet function and of the coagulation and fibrinolytic systems occur in association with diabetes mellitus.[1 2] The effect of the degree of metabolic control of diabetes on these tests of haemostasis has received little attention, though adrenaline-induced platelet aggregation[3] and factor VIII ristocetin cofactor activity[4] have been reported to be reduced when diabetics were intensively treated with diet and insulin. Recently, the sulphonylurea gliclazide was introduced to clinical practice in the United Kingdom. This drug has been claimed to reduce platelet retention and aggregation when given to patients with maturity-onset diabetes,[5 6] but studies have not distinguished between a specific drug-induced effect on platelet function and the possible influence of improved glycaemic control brought about by this sulphonylurea. We therefore assessed for one year the effects of diet and gliclazide on a panel of haemostatic tests in patients with non-insulin-dependent diabetes.

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Patients and methods

Fourteen newly diagnosed non-insulin-dependent diabetics completed the study. Table I gives their clinical details. Their mean age was 59·1 (range 42-70) years. Two patients had exudative retinopathy and two had symptoms of peripheral neuropathy at the time of diagnosis, the remainder having no symptoms or signs of vascular disease. Diabetes mellitus was diagnosed on the basis of a 50 g two-hour glucose tolerance test using the criteria proposed by Keen *et al.*[7]

All patients were seen at regular intervals by a dietitian and were educated to follow a conventional carbohydrate-restricted diet. After eight weeks they underwent a second glucose tolerance test. If the two-hour blood glucose concentration exceeded 6 mmol/l (108 mg/100 ml) gliclazide was prescribed in addition to the diet, the dose being adjusted to give optimal control of blood glucose concentrations. No other drugs were prescribed during the study. The patients were asked not to take aspirin-containing compounds and were given paracetamol as a substitute.

After informed consent had been obtained patients were reviewed at two-monthly intervals throughout the study, when non-fasting venous blood was removed using a 19-gauge butterfly cannula. Blood was obtained at the same time at each visit (one to two hours after breakfast).

Blood glucose concentration was measured by a glucose oxidase method, platelet count with a Coulter Thrombocounter, platelet retention to glass beads by a modification[6] of Salzman's method, factor VIII coagulant activity by a one-stage clotting assay,[8] and factor VIII-related antigen by immunoelectrophoresis.[9] Euglobulin clot lysis time was measured by the method of Blix[10] (results expressed as arbitrary units[11]), fibrinogen concentration by the method of Ratnoff and Menzie,[12] antithrombin III concentration by immunodiffusion,[13] heparin neutralising activity of platelet-poor plasma by a heparin-thrombin clotting time assay,[14] and β-thromboglobulin concentration using a radioimmunoassay provided by the Radiochemical Centre (Amersham, England). Results for factor VIII and antithrombin III were expressed as the percentage of the results obtained in a normal plasma pool from 20 healthy male controls. Serum gliclazide con-

TABLE I—*Clinical details of diabetics studied*

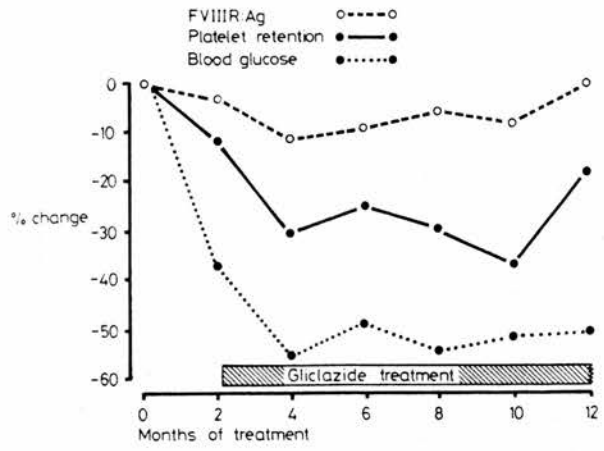
Case No	Age (years)	Sex	Complications	% ideal body weight* at:			Dose of gliclazide (mg/day)
				Onset	2 months	12 months	
			Treated by diet and gliclazide				
1	55	F		141	132	126	80
2	62	F		112	103	103	80
3	70	M		107	101	101	160
4	61	M	Peripheral neuropathy	114	111	120	240
5	61	F		156	141	147	160
6	42	F		100	99	103	240
7	55	M	Peripheral neuropathy	123	110	110	80
8	64	M	Exudative retinopathy	106	105	109	240
9	68	F		111	106	107	320
10	66	F		128	122	126	120
11	65	M	Exudative retinopathy	101	103	107	160
			Treated by diet alone				
12	56	M		103	102	105	
13	55	M		120	107	105	
14	47	M		110	111	118	

*Calculated using Metropolitan Life Assurance Co tables.

ations were measured by gas-liquid chromatography.[15] Platelet tion and euglobulin lysis times were measured immediately blood sampling; the remaining tests were performed on samples n and stored at −70 C.
sults were compared with pretreatment values using the Wilcoxon sign test for paired samples.

ults

ıe mean percentage ideal body weight for height at diagnosis 116·7 ± SEM 2·5%, and after two months' dietary treatment fallen significantly to 110 ± 2·5% ($p < 0·01$). By the end of the , however, it had increased significantly to 113 ± 3·4% compared the mean at two months ($p < 0·01$).
ree patients responded to dietary restriction alone and remained controlled at the end of the study (mean postprandial plasma se concentration 4·3 mmol/l (77 mg/100 ml)). After two months emaining 11 diabetics were not satisfactorily controlled by diet and gliclazide was introduced, daily doses ranging from 80 to ng/day. One patient withdrew from the study at the onset of zide treatment because of dyspepsia (superficial gastritis shown astroscopy), which also recurred when another sulphonylurea, ide, was given. Two patients had symptoms of hypoglycaemia, ı were abolished when the dose of gliclazide was reduced.
ble II summarises the laboratory findings in the 11 patients d with diet and gliclazide. After two months of diet alone icant reductions were observed in plasma glucose concentration actor VIII coagulant activity. After the introduction of gliclazide was a further significant fall in plasma glucose concentration. Platelet retention, factor VIII coagulant activity, factor VIII-related antigen, and plasma heparin neutralising activity were reduced significantly compared with pretreatment values. Mean serum gliclazide concentrations remained constant throughout the treatment period, and the remaining haemostatic tests showed no significant changes. When percentage changes compared with pretreatment values were plotted (figure) the changes in platelet retention and



Percentage changes in mean blood glucose concentration, platelet retention, and factor VIII-related antigen (FVIIIR:Ag) compared with pretreatment values in 11 diabetics treated with diet for 12 months and gliclazide from the second to the twelfth month.

TABLE II—*Laboratory results in the 11 diabetics treated with diet for 12 months and with gliclazide from two to 12 months. Values are means (± SEM)*

	Months of treatment						
	0	2	4	6	8	10	12
Blood glucose (mmol/l)	14·7 (1·5)	9·3† (0·8)	6·6‡ (0·7)	7·5‡ (0·6)	6·7‡ (0·5)	7·0‡ (0·7)	7·3‡ (0·7)
Serum gliclazide (mg/l)			5·3 (0·7)	5·0 (1·0)	5·7 (1·1)	4·8 (1·0)	5·3 (1·0)
Platelet retention (%)	35·5 (3·1)	31·3 (2·5)	24·7* (2·1)	26·7* (3·3)	25·0* (2·7)	22·3† (2·0)	29·2 (4·1)
Factor VIII-related antigen (%)	165 (16)	161 (19)	143* (22)	149 (24)	156 (23)	151 (29)	165 (34)
Factor VIII coagulant activity (%)	172 (20)	129* (14)	130* (8)	112† (10)	131* (10)	176 (27)	151 (28)
Heparin neutralising activity (U/ml)	0·21 (0·04)	0·16 (0·03)	0·14* (0·02)	0·11* (0·01)	0·14* (0·03)	0·12* (0·02)	0·13* (0·01)
Euglobulin lysis time (U)	13·2 (0·4)	12·1 (0·6)	12·6 (0·3)	12·7 (0·3)	12·9 (0·3)	12·7 (0·5)	12·4 (0·4)
β-thromboglobulin (μg/l)	56·2 (12·4)	68·9 (11·5)	43·4 (6·8)	77·9 (18·1)	64·2 (17·3)	63·2 (10·1)	44·1 (6·0)
Fibrogen (g/l)	3·00 (0·18)	2·72 (0·17)	2·94 (0·16)	2·81 (0·21)	2·70 (0·24)	2·95 (0·18)	2·77 (0·19)
Antithrombin III (%)	107·8 (4·6)	111·4 (4·5)	112·0 (5·8)	105·0 (4·6)	98·9 (4·7)	99·9 (5·6)	100·5 (3·9)

*$p < 0·05$, †$p < 0·01$ compared with pretreatment values.
‡$p < 0·01$ compared with values after two months' diet alone.
Conversion: SI to traditional units—Glucose: 1 mmol/l ≈ 18 mg/100 ml.

TABLE III—*Laboratory results in the three patients treated with diet alone*

	Case No	Months of treatment with diet alone						
		0	2	4	6	8	10	12
Blood glucose (mmol/l)	12	14·4	5·6	6·1	5·1	7·7	5·2	2·6
	13	10·1	5·0	4·5	4·0	3·5	3·2	4·0
	14	22·0	4·0	5·2	4·8	3·6	4·5	7·7
Platelet retention (%)	12	48·0	16·0	17·0	20·0	8·0	26·0	24·0
	13	17·0	27·9	25·0	24·0	17·0	8·0	20·0
	14	17·0	20·0	9·0	8·0	17·0	14·0	14·0
Factor VIII-related antigen (%)	12	147	105	88	116	104	98	126
	13	123	136	127	91	150	162	156
	14	202	100	112	103	109	171	175
Factor VIII coagulant activity (%)	12	175	195	140	86	102	113	98
	13	108	98	121	121	93	120	123
	14	219	126	135	155	140	128	163
Heparin neutralising activity (U/ml)	12	0·172	0·112	0·136	0·102	0·105	0·110	0·136
	13	0·194	0·120	0·218	0·204	0·092	0·128	0·124
	14	0·304	0·118	0·290	0·550	0·222	0·180	0·125

Conversion: SI to traditional units—Glucose: 1 mmol/l ≈ 18 mg/100 ml.

factor VIII-related antigen followed the same pattern as changes in postprandial plasma glucose concentrations.

Because of the small number of patients who were adequately controlled by diet alone, statistical analysis of this group was not possible. Nevertheless, reductions in mean platelet retention, factor VIII coagulant activity, factor VIII-related antigen, and heparin neutralising activity were observed (table III), while results of the remaining tests did not change.

Discussion

In this study satisfactory glycaemic control was achieved when gliclazide was added to the dietary treatment of newly diagnosed non-insulin dependent diabetics who were not adequately controlled with diet alone. Reduction of blood glucose concentrations was associated with changes in tests of haemostatic function—namely, a reduction in platelet retention, factor VIII coagulant activity, factor VIII-related antigen, and heparin neutralising activity. Smaller changes in these tests occurred after the two months of diet alone, which, with the exception of the change in factor VIII coagulant activity, failed to reach significance. Since the "ideal" weight was not achieved in all these patients, extending the dietary period might possibly have caused further falls in plasma glucose concentration with a corresponding improvement in these haemostatic tests.

Towards the end of the 12 months the results of the tests tended to return towards pretreatment values, whether the patients were treated by diet alone or by diet plus gliclazide. In the gliclazide-treated group mean drug concentrations remained constant, suggesting good compliance with treatment, but there was a significant increase in body weight and a slight rise in blood glucose concentrations at 12 months, which may imply that dietary habits deteriorated towards the end of the study.

Previous workers showed that treatment of maturity-onset diabetes with gliclazide resulted in reduction of platelet retention to glass beads and of adenosine-diphosphate-induced platelet aggregation.[5 6] In those studies, however, such treatment also produced an improvement in plasma glucose concentrations, and it is therefore uncertain whether the changes in platelet function were due to the drug or to improved metabolic control. The present study appears to favour the latter hypothesis. Treatment by diet alone resulted in improvements in certain haemostatic tests, and falls in plasma glucose concentration were paralleled by a corresponding reduction in factor VIII-related antigen and platelet retention.

The biological implications of such changes are uncertain. Previous studies have shown that haemostatic abnormalities tend to be most pronounced in patients with diabetic vascular complications.[1 2] If an abnormal haemostatic mechanism plays a part in the pathogenesis of diabetic angiopathies then a return of these tests towards normal might be expected to be beneficial. On the other hand, such abnormalities may be purely secondary to vascular damage. For example, in adequately controlled diabetes the highest concentrations of factor VIII-related antigen are generally found in diabetics with microvascular disease, but concentrations rise sharply during diabetic ketoacidosis and return towards normal after correction of the metabolic disturbance.[16] Factor VIII-related antigen is produced by vascular endothelial cells,[17] so it is possible that raised concentrations of this antigen during periods of poor diabetic control indicate vascular endothelial damage. Factor VIII coagulant activity, considered to be an acute-phase reactant,[18] is not synthesised by endothelial cells. Raised factor VIII coagulant activity may therefore represent tissue injury also caused by the disordered carbohydrate and lipid metabolism in uncontrolled diabetes.

The ability of plasma to neutralise heparin is partly due to a factor released from platelets (platelet factor 4). Plasma values of both heparin neutralising activity[19] and platelet factor 4[20] have been reported to be increased in diabetes. A proportion of heparin neutralising activity, however, is contributed by α-glycoproteins,[21] which are also raised in diabetes.[22] As plasma β-thromboglobulin and platelet factor 4 are released simultaneously from platelets[23] the most likely explanation from the present study for a change in heparin neutralising activity during treatment would appear to be a change in α-glycoprotein components.

In conclusion, the results of this study do not support the view that gliclazide per se exerts a beneficial effect on the haemostatic function of non-insulin-dependent diabetes. Nevertheless, control of plasma glucose concentrations by diet and gliclazide led to an improvement in some tests of coagulation and platelet function. Whether such changes lead to a reduction in diabetic vascular complications must await the results of controlled prospective clinical trials.

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